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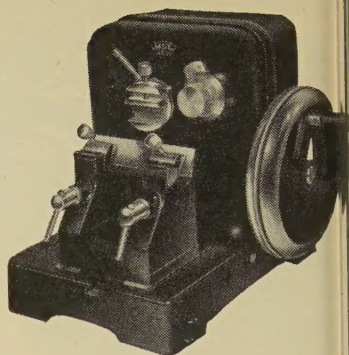
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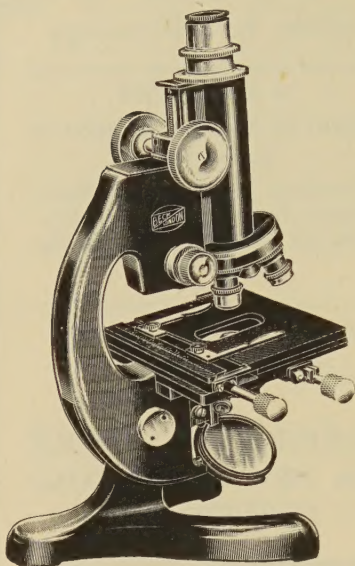
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Tests by Tissue Culture Methods on the Nature of Immunity to Transplanted Skin

BY

P. B. MEDAWAR

(From the Department of Zoology, University of Birmingham)

WHEN skin is grafted from one human being or one rabbit to another, a 'defence' mechanism is called into action which leads, in due course, to the complete destruction of the foreign grafted tissue. A quantitative analysis of the phenomenon (Gibson and Medawar, 1943; Medawar, 1944, 1945, 1946a, b) has shown that it conforms in broad outline with a reaction of actively acquired immunity. In other words, a human being at first submits to and later recovers from an 'attack' of foreign skin in much the same way as he recovers from an attack of measles: natural (i.e. ready-made) immunity is absent or ineffective; resistance develops in the course of exposure; and recovery is followed by a refractory or 'immune' state. This interpretation is not new, for certain critical tests bearing upon it were made by Peyton Rous in 1910; but it needs emphasizing, because Loeb (1921, 1930, 1945) has for many years denied that immunity in this technical sense has any important part to play in the organism's reaction against tissue grafted to it from other members of its own species.

More specifically, a skin homograft builds up a systemic reaction against itself at a rate which varies with the antigenic relationship between donor and recipient and with the quantity of foreign tissue that is grafted. A second homograft, transplanted from the same donor to the same recipient when the reaction against its predecessor is complete, survives for a few days in a vegetative condition in which cell division is partly or wholly suppressed, and then undergoes accelerated breakdown (Medawar, 1946a).

Skin transplantation immunity is thus easily recognizable *in vivo* by the regression of foreign homologous grafts and the refractory state which follows. If it conformed in detail, as it does in main outline, with the pattern of immunity created by bacterial and other crudely foreign antigens, then one might expect to find some immune body in the tissue or body fluids of an immunized animal which is inimical to the growth *in vitro* of the tissue responsible for generating the immune state. Many years' experience of tumour transplantation has led to the belief that no such factors exist, and it is hard to be convinced by the few tests of the hypothesis that have been said to have had a positive outcome (cf. Phelps, 1937). The purpose of the present paper is to reinvestigate the problem systematically, by taking advantage of newly devised methods that make possible the cultivation *in vitro* of adult skin.

PLAN AND METHODS

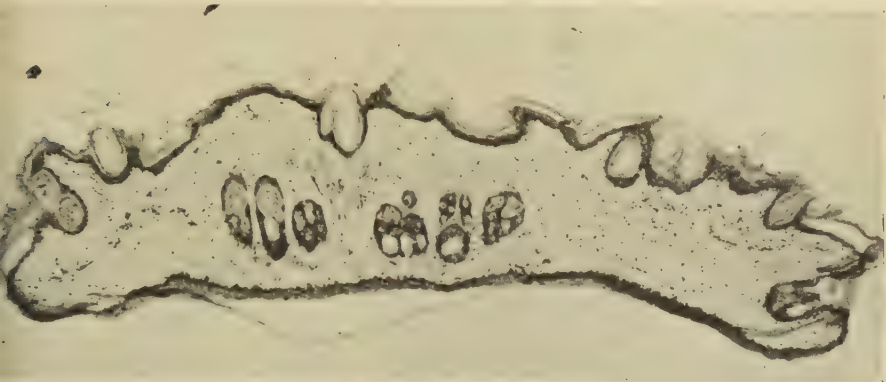
The tests to be described in this paper consist in the cultivation of adult rabbit skin epithelium in the presence of serum, tissues, and tissue extracts derived solely from a rabbit heavily and specifically immunized against it. Adult skin epithelium will proliferate and migrate promptly and vigorously when cultivated by flotation upon a stirred and properly aerated serous fluid medium (Medawar, 1948a). The advantages of fluid culture are its ease of execution, the rapidity with which cell division and migration begin and proceed, and the fact that the cells preserve their normal histological appearance and functional activity during cultivation (Text-figs. 1-5). Its only disadvantage is that, since migratory overgrowth or self-encystment takes the place of outgrowth as it normally occurs *in vitro*, histological analysis must, of necessity, turn upon the use of stained serial sections.

Each single test has made use of an independent pair of rabbits: a donor (D) and a recipient (R). In the initial immunizing operation, eight 'pinch' grafts, each about 10 mm. in diameter and together weighing 0.45-0.55 g., were transplanted from the thigh of D to the chest of R (Medawar, 1944, 1945). (In many trials the recipient was grafted on a second occasion from the same donor.) When the complete and long-standing necrosis of these immunizing grafts gave direct evidence of the completeness of immunization, but in no case earlier than the fifteenth day following their transplantation, a number of very thin 3 mm. $\times$ 3 mm. skin squares (minute 'Thiersch' grafts) were cut from a lightly vaselined area on D which had been toughened and rendered slightly hyperplastic by shaving 3 or 4 days beforehand (see Medawar, 1948a). The skin squares were thereupon cultivated for 4 or 8 days by flotation, raw side down, on serum freshly withdrawn from the median ear artery of R in dilatation (Medawar, 1946b), the serum being used either plain, or as an extractive for R tissues, or, most commonly, as a vehicle for the simultaneous cultivation of a variety of tissues from R. Although the D skin explants usually coalesced with at least some of the R tissue fragments cultivated with them, it was important in some special trials to make quite certain that coalescence and intimate tissue union should take place. In these special trials, therefore, R tissue fragments were glued to the dermal sides of the D skin explants with citrated and recalcified R plasma before cultivation began (see Medawar, 1948a).

Culture methods. Two culture methods were used: cultivation for 4 days in roller tubes, in a gas phase of air, or for 4-8 days in 'rocker flasks' of 250 ml. capacity in a gas phase of air-oxygen mixture (65-70 per cent. O₂). The design and use of the appropriate apparatus has been described in full elsewhere (Medawar, 1948a). The rocker-flask cultures were left undisturbed in the incubator for the run of each experiment. With roller-tube cultures the fluid part of the medium was replaced after the second day—with freshly withdrawn serum, if serum alone had been used, but with serum stored for 2 days in the refrigerator if the recipient animal had been killed at the beginning of the

experiment to provide 'immune' tissue explants. The main purpose of using high- O_2 rocker-flask cultures was to make certain of the continued functional survival of R spleen- and lymph-node tissue. Neither grows well, if at all, at low oxygen tensions (Parker, 1936, 1937).

Controls were achieved in two ways: either by the growth of D skin in normal homologous serum, tissues, and tissue extracts—i.e. in a medium derived



TEXT-FIG. 1. Exp. 18, Table 1. A 4-day roller-tube culture of donor skin in immune serum. Complete self-encystment (****) by migratory overgrowth. Ehrlich's haematoxylin and eosin. $\times 44$.

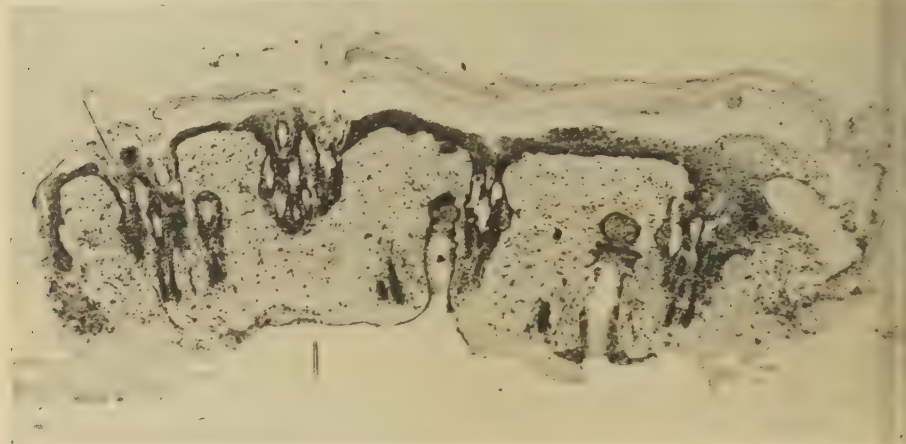
from a rabbit immunized neither against D cells nor any others; or by cultivating R skin in the same vessel as D skin in media which, being derived solely from R, should in theory be inimical only to the growth of cells from D. (The skin explants were distinguished from their companions by being cut to triangular shape.) Controls of these two types are adequate to reveal any non-specific action by the ingredients of the culture medium. It should be noted that a third type of control, the cultivation of skin from a second rabbit in media derived from a recipient specifically immunized against a first, is not acceptable; for although the skin immunity reaction is strongly donor-specific (Medawar, 1946a), the second donor might well happen to share with the first some antigens not also present in R. Some degree of immunity would in that event be directed against it.

In all except the earlier roller-tube experiments (Table 1, exps. 1-16), volumes of the chosen culture medium were mixed before use with 1 volume of streptomycin solution (200 u./ml.) in Ringer. In the rocker-flask tests (Table 2), 8 volumes of the culture medium were mixed with 1 volume of streptomycin solution and 1 volume of 5 per cent. glucose solution in water. With these exceptions, and with the substitution of Krebs-Ringer-bicarbonate for serum in two specific trials (Table 1, exps. 34, 35), the culture medium for skin was derived wholly from the animal immunized against it.

METHODS OF ANALYSIS

Three separate routine tests and analyses have been made on roller-tube explants of donor skin in immune media: (1) a test of their continued survival; (2) a measure of the frequency of cell divisions in the epidermis; and (3) a measure of the migratory activity of the epithelium as a whole. Rocker-flask cultures in a high- O_2 gas phase were used mainly for the histological analysis of donor skin and immune mesenchymal tissue growing in intimate union.

1. *Survival Test.* So much of importance turns upon whether donor skin survives cultivation in immune media that the diagnosis of survival has no



TEXT-FIG. 2. Exp. 7a, Table 2. A 4-day rocker-flask culture of donor skin in immune serum containing lymph-node fragments, which have not attached themselves to the skin explant (contrast Text-fig. 9). Note the vigorous epidermal proliferation and the relatively subdued migratory activity. Ehrlich's haematoxylin and eosin. $\times 44$.

been allowed to rest on histological evidence alone. Instead, each D explant was duplicated; one was reserved for histological analysis and mitotic counting (see below), and the other tested for its continued survival by the simple process of grafting it back to a large raw area on the animal from which it originally came. Not less than two such tests of survival have been done for each type of medium to which the roller-tube explants were subjected (Table 1, col. 5). The test has been described in full in earlier papers (Medawar, 1947, 1948a).

2. *Mitotic Activity.* The other member of each pair of D skin explants was rinsed in Ringer, fixed in Bouin's solution, and embedded in wax. Alternate sections from strips of 10 taken at 5 evenly spaced vertical levels through the block were stained with Hance's variant of Heidenhain's haematoxylin. All the mitoses in the 5 alternating 8μ sections from the strip of median level were counted and averaged, the figure entered in column 6 of Table 1 representing the mean number of mitoses per median 8μ section divided by the width of the explant in millimetres.

3. *Migratory Activity.* A median section stained with Ehrlich's haematoxylin was marked for degree of epidermal migration in accordance with the scale which follows (Table 1, col. 7):

* Overgrowth incipient: the 'shoulders' of the explant rounded off.

** Overgrowth about 1/3rd complete.

*** Overgrowth about 2/3rds complete.

**** Complete self-encystment (cf. Text-figs. 1, 3, 4, 5).

RESULTS: ROLLER-TUBE CULTURES

The results of 37 tests are summarized by Table 1 and amplified by the notes given below, which correspond to the references in column 4. The use



TEXT-FIG. 3. Tissue coalescence test. A 4-day roller-tube culture of donor skin to the under side of which small fragments of kidney medulla have been glued with immune plasma. Culture medium: immune serum. Epidermal and medullary epithelium between them have brought about complete self-encystment of the explant. Ehrlich's haematoxylin and eosin. $\times 40$.

f streptomycin at a final concentration of 20 u./ml. in the culture medium was adopted from exp. 17 onwards. The use of minute 'Thiersch' grafts as explants (mean diameter after fixation: 2.9 ± 0.1 mm.) was not adopted until exp. 14; small 'pinch' grafts, much thicker at the centre and somewhat broader (4.0 ± 0.2 mm.) had been used until then, and this probably accounts for the fact that none of the explants quite achieved complete self-encystment (****) in 4 days. The total weight of the immune tissue explants added to the culture medium in various experiments did not exceed 4 mg.; each was cut into a cube not exceeding, and so far as possible not much less than, $1\frac{1}{2}$ mm. in length of side. In all the experiments described in this paper, the lymph-node explants were taken from the axillary node receiving the lymphatics from the side of the chest carrying the immunizing homografts.

TABLE I. *Roller-tube cultures: the results of 37 tests of the growth of donor skin explants in media of various types derived from rabbits specifically immunized against them.*

The explants have been marked for survival by the transplantation test (see text), and for mitotic and migratory activity. The entries in column 4 refer to the Notes in the text.

No.	Donor→ Recipient	Culture medium	Notes	Survival	Mitoses	Migra- tion
1	396→397	Immune serum	..	*	..	***
2	401→402	Immune serum	..	*	0·6	**
3	411	Normal homologous serum (from 413)	..	..	1·2	**
4	411→412	Immune serum	..	*	1·2	*
5	416	Normal homologous serum (from 413)	..	..	1·0	***
6	416→417	Immune serum	..	*	0·6	***
7	421	Normal homologous serum (from 416)	..	..	0·7	**
8	421→422	Immune serum	..	*	0·7	**
9	426→427	Immune serum	..	..	0·9	*
10	426→427	Immune serum + lymph-node ex- plants	..	*	1·5	**
11	431→432	Immune serum	..	..	1·1	**
12	431→432	Immune serum + lymph-node ex- plants	..	*	..	..
13	436→437	Immune serum + spleen explants	..	*	..	..
14	442→443	Immune serum + spleen explants	..	*	1·8	**
15	445→447	Immune serum + liver explants	..	*	0·6	*
16	451→453	Immune serum + liver explants	..	*	0·4	**
17	459→460	Immune serum + leucocytes	a	*	1·1	***
18	461→462	Immune serum (adjuvant immuniza- tion with chicken plasma)	b. Text- fig. 1	*	0·9	****
19	469→470	As exp. 18	b	..	0·7	****
20	471→472	Immune serum + leucocytes	a	*	1·2	**
21	473→474	Immune serum as extractive for liver and spleen tissue; adjuvant immu- nization with chicken plasma	bc	*	0·9	***
22	475→476	Immune serum + spleen explants	..	..	1·4	****
23	475→476	Immune serum + lymph-node ex- plants	Text- fig. 4	..	1·3	****
24	478→479	Immune serum as extractive for liver and spleen tissue, with liver and spleen explants; adjuvant immuni- zation with chicken plasma	bc	*	0·7	***
25	481→482	R skin in D serum + liver and spleen explants; 'reverse' test	d	*	0·8	****
26	488→489	As exp. 25	d	*	1·1	****
27	519→520	Immune serum + kidney medulla ex- plants	..	*	1·0	***
28	527→528	As exp. 27	..	*	0·7	****
29	532→533	20 per cent. defibrinated whole blood in serum + blood-clot explants	e	*	1·2	****
30	537→538	As exp. 29	e	*	1·0	****
31	539→540	As exp. 29	e	*	2·2	****
32	545→546	Immune serum anaerobically	f	*	0	0
33	550→551	Immune serum anaerobically	f	*	0	0
34	550→551	Ringer-bicarbonate as extractive for kidney and liver tissue	c	*	2·1	****
35	560→561	Ringer-bicarbonate as extractive for kidney and spleen tissue	c	*	3·9	****
36	604→605	100 per cent. defibrinated whole blood; adjuvant immunization with heterologous cells	eg	*	1·0	****
37	608→609	As exp. 36	eg	*	1·1	****

Notes on Table I

(a) *The use of leucocytes* (exps. 17, 20). Leucocytes were present in the immune serum at $2\frac{1}{2}$ times their physiological concentration, having been separated from 6 to 8 ml. citrated whole blood by repeated slow spinings and then resuspended in 3 ml. serum. The serum reserved a strong cellular opalescence during cultivation, though a majority of the leucocytes agglutinated and formed compact cell clusters.

(b) *Adjuvant immunization with chicken plasma* (exps. 18, 19, 21, 24). The recipients were injected with 2 ml. chicken plasma 12 days before and 4 days after the transplantation of the immunizing dose of homografts, the first injection being intradermal and the second intravenous. The antigen dilution titre of precipitins in the R serum exceeded 1,000 when the culture tests were begun 12 days after the second injection.

(c) *Tissue extractions* (exps. 21, 24, 34, 35). About 1 g. of each type of tissue was chopped into a fine mush with scissors and extracted with 10 ml. serum (exps. 21, 24) or 10 ml. Krebs-Ringer-bicarbonate (exps. 34, 35). In each case 2.7 ml. of the supernatant fluid, clarified by spinning, served as the culture medium.

(d) *'Reverse' tests* (exps. 25, 26). The skin of the immunized recipient was cultivated in serum and tissue fragments derived from its donor.

(e) *Defibrinated blood* (exps. 29, 30, 31, 36, 37). It proved best to dilute the defibrinated whole blood with 4-5 volumes of its own serum.

(f) *Anaerobic cultivation* (exps. 32, 33). These experiments, though entered here for ease of reference, made use of the anaerobic culture apparatus described by Medawar (1947), and not of roller tubes. In this apparatus the explants were incubated for 4 days at 38°C . in 5 ml. serum under an atmosphere of H_2 from which the last traces of O_2 had been removed by catalysis with heated palladium (indicator: methylene blue). Skin epithelium incubated anaerobically neither moves nor proliferates in any degree; hence the zero entries in the last two columns. The survival test shows, however, that in immune serum as in normal serum (Medawar, 1947), the skin cells remain alive and resume normal growth and activity when oxygen is restored to them.

(g) *Adjuvant immunization with a cellular antigen* (exps. 36, 37). A fine suspension in Ringer of about 100 mg. adult mouse spleen, lymph node, and submaxillary gland fragments was injected intradermally and intraperitoneally into R on the day of its receiving the immunizing grafts from its homologous donor. In exp. 37 the heterologous cell suspension was, in addition, injected intradermally into the graft donor area of D immediately before the immunizing grafts were cut from it for transplantation to R.

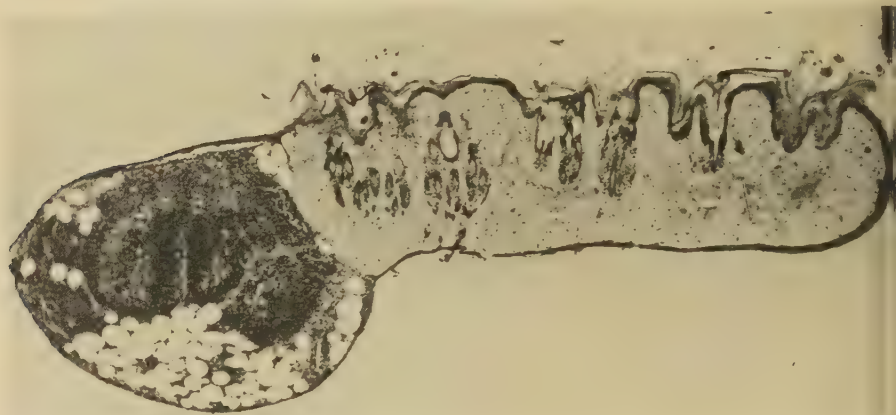
Tissue Coalescence Tests

Special interest attaches to an additional and independent series of roller-tube cultures in which fragments of 'immune' tissue were glued on to the dermal surfaces of the skin explants before cultivation began (see above). Text-figs. 4 and 5 illustrate the complete encapsulation of lymph-node fragments by skin epithelium that has migrated from the donor explants. In Text-fig. 5 the very pronounced thickening of the epibolic skin epithelium in the immediate neighbourhood of the encapsulated explant shows that, so far from its having had any inhibitory action, the node tissue seems actually to have stimulated the growth of the adjacent epithelial cells. Text-fig. 3 in low power and Text-fig. 8 in higher power illustrate complete confluence between donor skin epithelium and epithelium from the immunized rabbit's kidney medulla. It is hard to tell where the one ends and the other begins.

The tests using lymph-node explants (and others using spleen) are not decisive, because cultivation in a gas phase of air is not adequate to maintain the complete functional survival of tissues of these two types. The experiments to be described in the next section were done mainly to repair this shortcoming.

RESULTS: ROCKER-FLASK CULTURES

The cultures to be described here were done in 250 ml. gas perfusion rocker flasks containing the following culture ingredients: (a) 4–15 ml. immune serum containing streptomycin and glucose at final concentrations of 20 u./ml. and 0.5 per cent. respectively; (b) two square or rectangular donor skin explants of the same size as those used in roller tubes; (c) two skin explants from the recipient, cut to triangular shape for ease of identification and serving as controls against the possibility of any non-specific action by the ingredients of the medium; and (d) up to 88 mg. immune spleen or lymph-node tissue.



TEXT-FIG. 4. Exp. 23, Table 1. A 4-day roller-tube culture in immune serum containing lymph-node fragments, one of which has by chance attached itself to the edge of the skin explant and become encysted by epidermal epithelium. Ehrlich's haematoxylin and eosin. $\times 37$.

(mainly the former) cleanly chopped into cubical fragments of the size habitually used in tissue cultivation.

The flasks were perfused with one measured volume of filtered cylinder O_2 at the timed rate of 100 ml. per minute, giving a final concentration of 65–70 per cent. O_2 , and then set to rock for from 4 to 8 days without further attention in a dry-air incubator at $38^\circ C$. At the end of the experiment the pH of the culture medium was tested with the glass electrode and the cultivated tissues were fixed in formal- $HgCl_2$ for histological analysis. Independent survival tests were done in only two cases, with positive results. Both spleen and lymph-node fragments showed obvious proliferation under these conditions of culture (Text-figs. 6, 7), but the results from the cultivation of spleen were characteristically variable (cf. Parker, 1936, 1937). In most cultures the medium became strongly opalescent with mononuclear cells that had migrated from the spleen explants during the first day or two of cultivation. The occurrence or failure of this response was in no way correlated with the degree of survival of the spleen explants as judged by their histological appearance.

at the end of the experiment. Cells migrating from the lymph-node explants showed a strong tendency to infiltrate the dermis of donor and control skin explants alike and to bring about some degree of collagen dissolution.

The behaviour of the skin explants was also variable: there were occasionally greater differences among the members of the two pairs than between them. For this reason many more cultures (Table 2) had to be done than would otherwise have been necessary. In high-O₂ media the migratory activity of the skin epithelium was far less pronounced than with cultivation in a gas phase of air, and complete self-encystment was achieved more slowly and less often. Cell division, on the other hand, was rapid and led to the formation of a deeply stratified epidermis (Text-fig. 2).

TABLE 2. *Rocker-flask cultures in a high-O₂ gas phase*

Summarizing the various culture-medium volumes, culture periods, and tissue dosages used in control cultures in autologous media (group A) and in experimental cultures in immune media (group B).

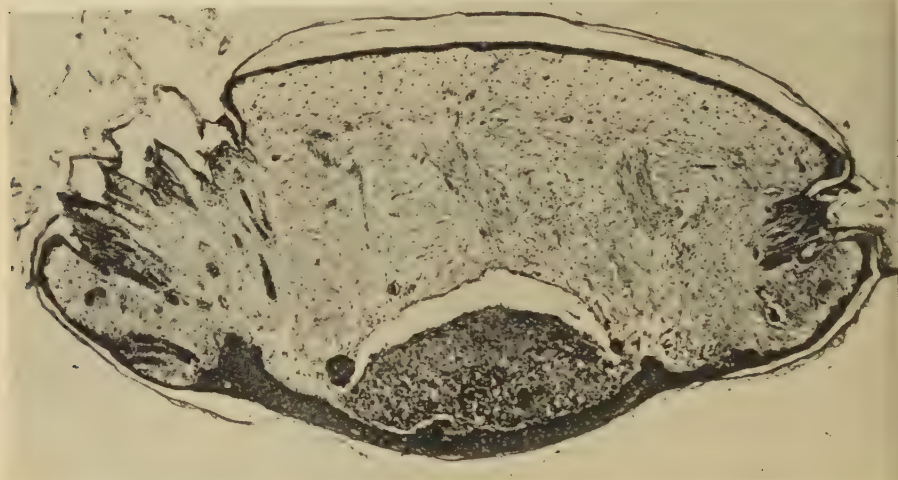
No.	Donor→ Recipient	Volume of medium, ml.	Days of cultivation	Tissue	Final pH	Notes
A. 1	669	10	4	72 mg. spleen	7·81	0·2 per cent. NaHCO ₃ in culture medium.
2	672	10	4	46 mg. spleen	7·45	
3	679	10	4	36 mg. spleen	7·65	
4	680	6	4	55 mg. spleen	7·05	
5	685	4	4	33 mg. spleen	7·60	
6	685	6	4	37 mg. spleen	7·60	
B. 7a	651→652	10	4	50 mg. node	..	Text-figs. 2, 9.
7b	651	10	4	50 mg. node	..	Normal homologous serum from 647; no glucose.
8	653→654	12	4	30 mg. spleen	..	No glucose.
9	657→658	10	6	25 mg. spleen	7·65	Glucose 1 per cent.
10	659→660	11	5	80 mg. spleen	..	Recipient not fully immune.
11	663→664	10	4	36 mg. spleen	7·25	Medium infected.
12	665→666	10	4	38 mg. spleen	7·05	
13	667→668	8	4	51 mg. spleen	7·30	
14	686→687	10	8	61 mg. spleen	6·65	Medium infected.
15	690→691	15	8	47 mg. spleen	7·20	
16	694→695	15	8	88 mg. spleen	6·70	
17	696→697	10	8	68 mg. spleen	6·78	
18	700→701	15	8	36 mg. spleen	7·25	
19	706→707	15	8	Serum alone	7·15	
20	710→711	15	8	74 mg. node	7·42	
21	712→713	15	8	55 mg. spleen	7·45	
22	724→725	6	4	36 mg. spleen	7·21	
23	726→727	8	4	45 mg. spleen	7·19	
24	728→729	6	4	49 mg. node	7·46	

Results. The cultures which were analysed are set out in summary in Table 2. There was no appreciable difference between the experimental cultures and the controls run in media of autologous origin.

In the experimental group the donor skin explants were quite clearly superior to their controls (i.e. the skin explants taken from the immunized recipient itself) in exps. 15 and 18, and clearly inferior in exps. 12, 14, 22, and

23. (In exp. 14, as a result of infection, the growth of both skin pairs was very poor.) There was no ground for supposing that these differences were anything but fortuitous in origin.

There was almost invariably some degree of union between the skin explants and the fragments of spleen or lymph-node tissue cultivated with them. Text-fig. 9 illustrates a particularly intimate union between strongly proliferating donor skin epithelium and an immune lymph-node fragment, almost wholly encapsulated by it. Mitotic figures, everywhere abundant,



TEXT-FIG. 5. Tissue coalescence test. As Text-fig. 4, save that in this case a fragment of lymph node was deliberately glued on to the dermal surface of the skin explant, where it has now undergone complete epidermal encystment. The node tissue is now necrotic, but the epithelium in its neighbourhood is thicker than it is elsewhere. Ehrlich's haematoxylin and eosin. $\times 44$.

were not less frequent in the epibolic epithelium abutting immediately against the node tissue. In short, the entire series of experiments affirms that the mitotic and migratory activity of skin epithelium was in no way influenced by cultivation in media, and in intimate association with tissues, derived from a rabbit heavily immunized against it.

OTHER SEROLOGICAL TESTS

It has already been established (Medawar, 1946*b*) that the grafting of skin from one rabbit to another does not elicit the formation of red-cell isoagglutinins. It might conceivably elicit the formation of agglutinins for cell suspensions of donor skin epithelium itself; and to complete the present series of *in vitro* tests the possibility was examined as follows.

About 1 cm.² of the thinnest possible shavings from the vaselined hyperplastic skin of a donor rabbit was incubated for one hour in a Seitz-filtered 0.5 per cent. solution of commercial trypsin powder in Ringer-bicarbonate at

pH 7.6 (Medawar, 1941). The shavings were thoroughly rinsed in Ringer, and from each one the dermal layer was lifted off with fine forceps, leaving behind a pure epidermal sheet which transplantation tests have shown to be

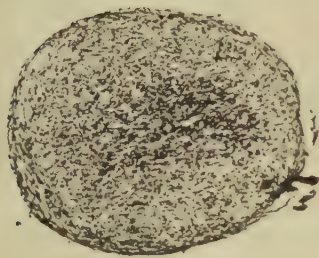


FIG. 6

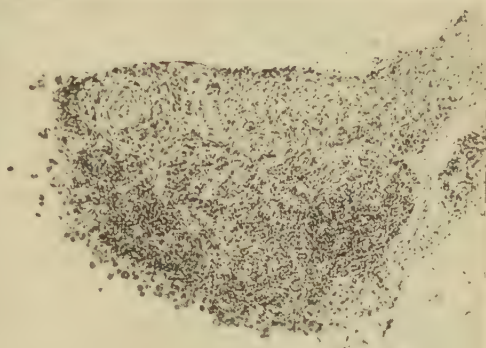


FIG. 7

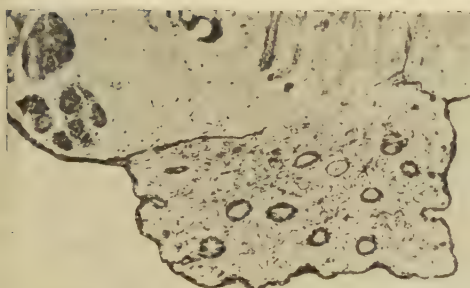


FIG. 8

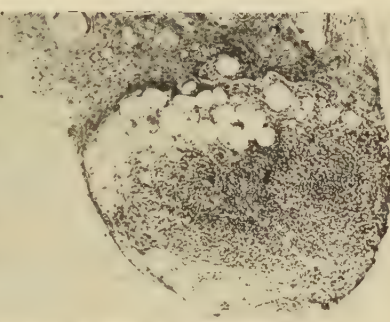


FIG. 9

TEXT-FIG. 6. Illustrating the characteristic appearance of a 4-day spleen fragment cultivated in the high- O_2 gas phase of a rocker flask. Ehrlich's haematoxylin. $\times 70$.

TEXT-FIG. 7. As Text-fig. 6: a lymph-node fragment.

TEXT-FIG. 8. See Text-fig. 3: an illustration in higher power of the coalescence of donor skin epithelium with 'immune' kidney medulla epithelium to form a continuous sheet. Ehrlich's haematoxylin and eosin. $\times 53$.

TEXT-FIG. 9. Exp. 7a, Table 2. Portion of a 4-day rocker-flask culture in immune serum containing lymph-node fragments, one of which has attached itself to the margin of the culture and undergone complete epidermal encystment. Note the strongly proliferating epithelium in its neighbourhood, and the complete failure of the 'immune' node tissue to affect its growth adversely. Heidenhain's haematoxylin.

$\times 53$.

still living (Billingham and Medawar, 1948). The sheets were flattened down on their vaselined cuticular surfaces and then smoothly and firmly scraped to separate the cells of the deeper layers from those of the cuticle. The clumps so formed were rendered into a fine suspension in Ringer by sucking them

repeatedly in and out of a fine-bore pipette. The suspensions were then mixed in agglutination tubes with $\frac{1}{2}$ or 1 volume of serum from a rabbit specifically and effectively immunized against the donor skin. Five such tests, run for 3–6 hours at body temperature and overnight at room temperature, were uniformly negative in outcome, and the cell suspension which in due course settled in the bottom of the agglutination tubes could be evenly redispersed by light tapping or shaking.

The presence of precipitins for skin extracts was tested simultaneously by the ring method, a water-clear extract being secured by freezing thin skin slices with CO₂ snow, cutting them into 15 μ sections on a freezing microtome, grinding the sections so cut in a mortar under Ringer, and then spinning down the collagenous and cuticular debris. No ring of precipitation developed between immune serum and the supernatant skin extract, and the subsequent mixing of the two solutions with further incubation produced no turbidity perceptible by direct or oblique illumination.

DISCUSSION

The experimental results make it clear that immunity to foreign homologous skin grafts is not demonstrable by an *in vitro* reaction of any type described in this paper. Notwithstanding statements that have from time to time been made to the contrary, they justify the provisional conclusion that the occurrence of free serum antibodies is not a *sufficient* explanation of the destruction of foreign homologous tissue. The tests involved the cultivation of not more than 5 mg. donor skin for up to 8 days in so much as 15 ml. of serum and 88 mg. of mesenchymal tissue derived solely from a rabbit specifically and demonstrably immunized against it. Within these limits the experimental results are quite decisive: skin epithelium survives, moves, and proliferates with unaffected vigour in these presumptively immune media.

The results are consistent with those of Harris (1943), which have not yet been fully reported on; and, also, with what is known of the relationship between the regression of tumour homografts and the occurrence in their hosts of antibodies revealed by complement fixation. Actively induced complement fixing iso-antibodies appear in the sera of rabbits bearing a variety of transplanted tumours (Friedewald and Kidd, 1945) and they react *in vitro* with the fine particulate matter thrown down from saline tissue extracts by spinning at about 20,000 *g* for 2–3 hours. An antibody elicited by at least one such tumour, the Brown-Pearce carcinoma, has since been shown to be specific to the tumour in question (MacKenzie and Kidd, 1945; Kidd, 1946). The lack of correlation between tumour regression and specific antibody titre makes it clear, however, that 'factors other than the specific antibody are probably responsible for regression of growth in the majority of cases' (Kidd, 1946).

Two recent attempts have been made to elicit the formation of iso-antibodies by injecting extracts or homogenates of foreign homologous tissues together with toxins of known antigenic power (Schwentker and Comploier,

1939; Hecht, Sulzberger, and Weil, 1943). In neither case was the homologous tissue by itself effective.

Four possibilities that indicate the limitations of the present preliminary analysis may now be briefly discussed. First, it is possible that cultivated tissues will survive for long periods, but not indefinitely, in media derived from animals immunized against them. What is known already about the tempo and vigour of the immune reaction *in vivo* gives one no ground for supposing this to be true. A second possibility is that the dosage relationships between the cultivated tissues and their media were inappropriate to the demonstration of an immune reaction. When a skin graft weighing about 0.05 g. is transplanted to a 2 kg. rabbit, it 'competes' with 40,000 times its own mass of native tissue. The corresponding dosage ratio for the tests *in vitro* reported here has not exceeded 20 at the outside. This second possibility will therefore be investigated further, though it depends upon an interpretation of the immune reaction—in terms of a theory of 'competition'—which is supported only by perhaps remote analogies with the behaviour of micro-organisms.

A third possibility, which preliminary tests suggest to be the one most worthy of further inquiry, is that immune media affect skin epithelium *in vitro* only in such a way as to expedite its breakdown on subsequent grafting. Woglom's (1933, 1937) experiments with transplanted rat sarcomata support this view, and Kidd (1946) has found that a preliminary incubation of Brown-Pearce tumour tissue in media containing its specific antibody reduced or suppressed its growth on transplantation. Finally, it may be that the hypothetical antibodies are cell-bound, and that an accessory immune mechanism is needed to bring them into action, viz. the destruction of the host's antibody-containing cells in the immediate neighbourhood of the graft. (The regression of skin grafts is accompanied by the wholesale destruction of such native lymphocytes and other leucocytes as may have penetrated them: cf. Medawar, 1944.)

The biological significance of the tissue homograft reaction deserves some mention. It is widely believed that tissue transplantation, like blood transfusion, is an act that has no counterpart in nature. This is clearly untrue: the mammalian foetus is a tissue homograft, though it is normally protected from the consequences of that fact by its strictly independent circulation. (The behaviour of skin homografts in the anterior chamber of the eye is to some extent a model of the mother-foetus relationship: such grafts, if not vascularized, are not destroyed: cf. Medawar, 1948*b*.) It is at present thought likely (cf. Penrose, 1946; Kalmus, 1947) that a variety of foetal and placental abnormalities are to be attributed to immunological incompatibilities between foetus and mother rather than, for example, to endocrine disorder. If this view is correct, the interpretation of some forms of foetal abnormality is likely to turn on the analysis of the tissue homograft reaction.

Text-figs. 1-5 are from photographs taken by Mr. D. A. Kempson. The

cost of the experimental animals used in this work was met by a grant from the Medical Research Council; of the special apparatus, by a grant from the Department of Plastic Surgery, Oxford University.

SUMMARY

The transplantation of skin from one rabbit to another elicits a reaction that conforms in main outline with that of an actively acquired immunity. The experiments described in this paper were designed to test the hypothesis that the regression of such grafts is secured by the action of antibodies demonstrable *in vitro*.

Skin from adult rabbits has therefore been cultivated in the presence of serum and growing mesenchymal tissues derived solely from rabbits heavily and specifically immunized against it.

Immune sera and tissues are without effect on the survival, cell-division frequency and migratory activities of explanted skin, and agglutinins for epidermal cell suspensions are not demonstrable in immune sera.

With certain stated qualifications, it has therefore been concluded that the occurrence of free antibodies is not a sufficient explanation of the regression of skin homografts *in vivo*.

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Budding and the Reproductive Cycle of *Distaplia*

BY

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THE process of budding and the production of tadpole larvae in *Distaplia* have long been of interest and have been the subject of many contributions. At the same time, while a great deal is now known about certain phases of the developmental cycle, other parts have remained irritatingly obscure, so much so that Julin (1896), for example, concluded that only tadpoles produced buds and that colonies were formed from tadpoles, with buds, that had failed to escape from the colonial mass. Studies to date have been made on one species, *Distaplia magnilarva*, and in relation to the nature of the tadpole and its metamorphosis, its production of buds, and the development, as distinct from origin, of buds in the adult colony. The present account is an attempt to describe the whole developmental cycle, based mainly on abundant material of *D. bermudensis* collected from the Gulf coast of Florida, with comparative material of *D. rosea*, *D. garstangi*, *D. clavata*, and *D. magnilarva*.

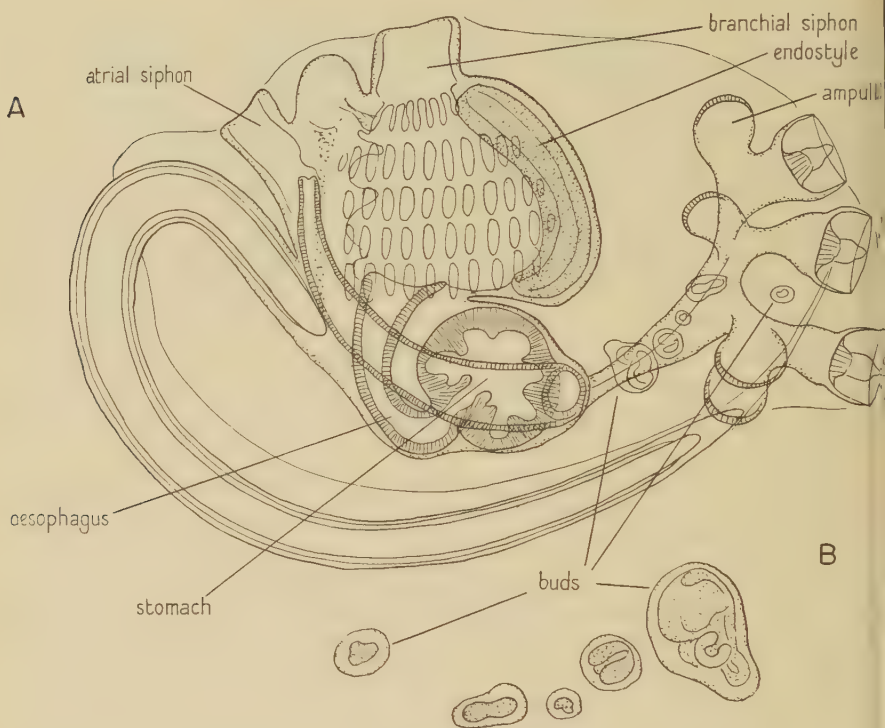
The most recent study, known only after the present investigations were completed, is that of Brien (1939) and is based upon *D. magnilarva*. It concerns solely the process of budding in the larvae, and the general conclusion is similar to that of Julin that the buds produced by the tadpole larvae give rise directly by continued budding to all the zooids comprising a colony. This appears to be a misinterpretation of the process occurring in the larva and the result of failure to discover any other source of buds from later stages. Such failure is by no means the result of lack of effort, and there is no doubt that both Julin and Brien made an intense and competent search for an origin of buds from adult zooids. The reason lies more with the nature of the species examined, and without doubt, had the present investigation been confined to a study of *D. magnilarva* and *D. rosea*, the problem would have remained unsolved. This question will be discussed further in the last section of this paper.

The present account is divided into three parts, representing three phases of the life-cycle. Any one of the three might have been the commencement since we are dealing with a cycle without a real beginning or an end. As a somewhat arbitrary starting-point the attached and newly active oozoid has been taken, with the first section consisting of the growth of zooids to sexual maturity involving successive asexual generations, together with the general development of buds up to and beyond the active functional state. The second

section concerns the development and nature of the tadpole larva, while the third concerns the regression of old zooids, the origin of buds from such zooids, and the first stages of morphogenesis in the bud. A comparative discussion is left to the end.

PART I. ORIGIN, NATURE, AND DEVELOPMENT OF ZOOIDS

This part is divided into three subsections, one concerning observations on *Distaplia rosea*, one on *D. bermudensis*, and a comparative section.

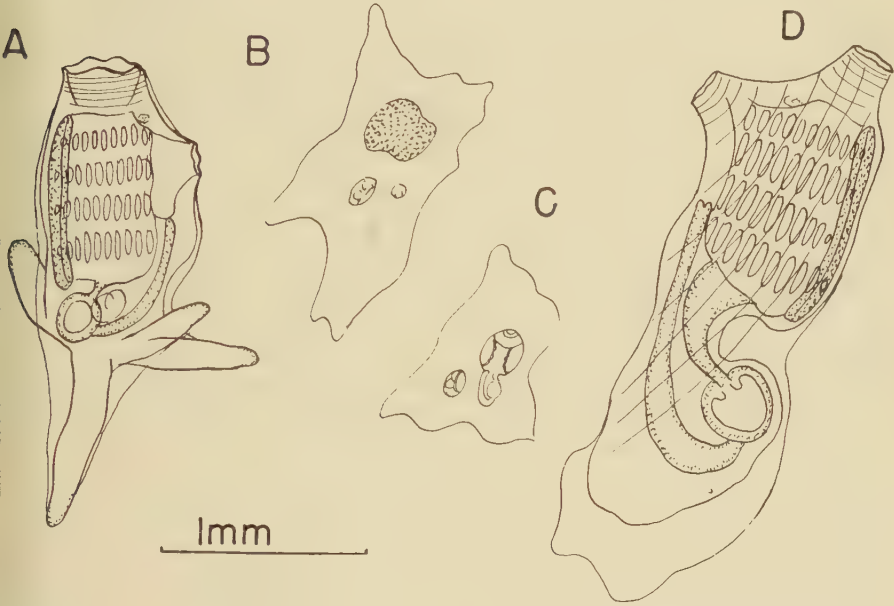


TEXT-FIG. 1. A. Tadpole of *D. rosea*, showing almost equivalent development of permanent and transient structures and series of buds. B. Bud series, enlarged and in reversed order.

Distaplia rosea

As an introduction, the tadpole larva is shown (Text-fig. 1A) not to emphasize the tadpole structure as such but the more permanent structures. These are more readily defined in a negative sense and comprise the larva with the exception of the tail, sensory vesicle, and the three anterior adhesive organs. This leaves the ascidiozooid with its siphons, endostyle, branchial sac, intestinal loop and heart, together with four epidermal ampullae and a number of buds enclosed within the larval test. In the example shown five buds of varying size are present.

After settling, the tadpole structures are resorbed and the oozoid becomes functional as such, and is shown in Text-fig. 2A. The four ampullae survive as the permanent organs of attachment after the disappearance of the temporary adhesive organs. During the active life of the oozoid the buds do not grow to any appreciable extent. After a certain period, to be measured in days in the case of *D. rosea*, weeks in the case of *D. bermudensis*, the oozoid undergoes resorption and finally disintegrates within its test. Coinciding with this



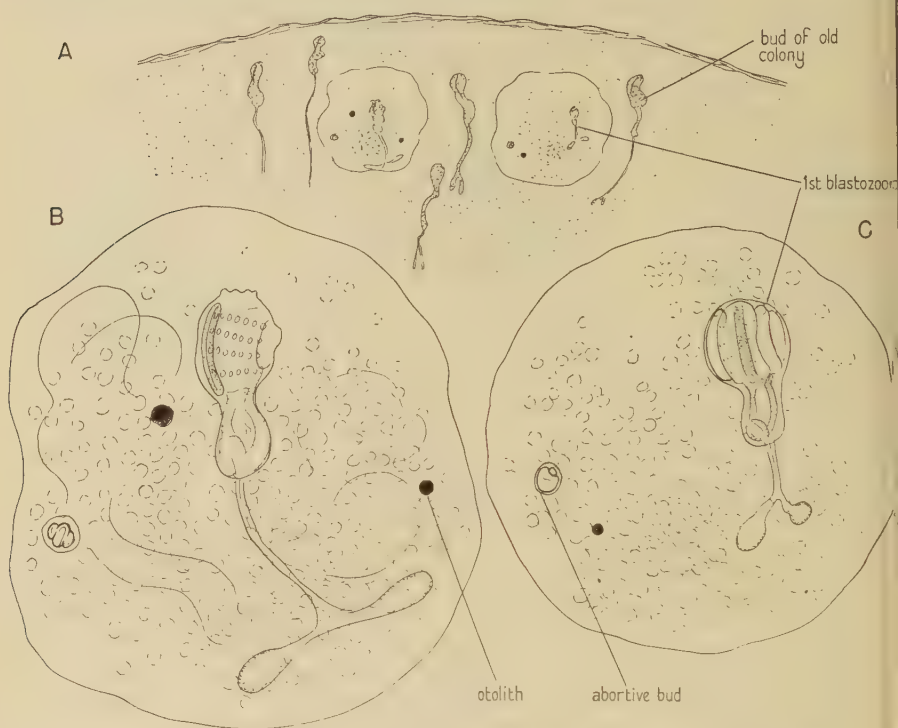
TEXT-FIG. 2. A. Fully metamorphosed oozoid of *D. rosea*. B. Resorbed oozoid and two surviving buds. C. Two buds of first blastozoid generation, one developing and one abortive. D. Fully developed first blastozoid.

resorption, the largest of the buds grows rapidly and the first blastozoid replaces the oozoid. In none of the many cases observed did the second generation ever consist of more than the one active blastozoid. Usually a second bud, apparently arrested at an early stage of development, can be seen, but with no sign of the remaining and smaller buds originally contained in the larval test. The following table expresses the duration of the various phases at 20° C. kept in finger-bowls submerged in running seawater.

Duration of phases of cycle at 20° C.

Active free-swimming period of tadpole	.	.	.	.	2 hours
Period from tadpole stage to functional oozoid	.	.	.	.	5 days
Survival of functional oozoid	.	.	.	.	16-24 days
Period of resorption or degeneration of oozoid	.	.	.	.	12-24 hours
Subsequent developmental period of first blastozoid	.	.	.	.	10-15 days
Survival of first blastozoid	.	.	.	.	8-10 days

The first blastozoid differs little from the established oozoid. It is somewhat larger, and while the gill slits remain unchanged as four rows of definitive stigmata as in the oozoid, the gut loop is considerably longer and better displays the divisions and appearances of that of mature zooids. Neither oozoid nor first blastozoid shows any observable sign of gonads. The survival periods for the oozoid and blastozoid, as indicated above, may be



TEXT-FIG. 3. A. Part of upper surface of a corm of *Distaplia rosea* after zooids resorbed and surface smooth, containing developing blastozoids and two incorporated young colonies resulting from retained tadpoles. B, C. The same two second-generation colonies enlarged to show in each case a developing first blastozoid, an abortive blastozoid, and remnants of the sense organs and ampullae of the oozoid.

abnormally brief, for aquarium conditions at their best may be none too good and it is possible that at lower temperatures, with more abundant food organisms, and the purer water of the natural environment, both forms might survive for a much longer period. What does appear to be established, however, is the comparative instability of the active zooid and that it does have a limited existence, one generation of zooids replacing another and being above all dependent for their own growth and development upon the degeneration products of the resorbing generation.

Julin (1896), working mainly with *D. magnilarva* and failing to find the source of new buds in old colonies, suggested that buds were replenished

from tadpoles produced within the colony by the mature zooids. He brought forward no positive evidence for this and Daumezon (1909) rejected the hypothesis on *a priori* grounds alone. However, in one of several small but fully mature colonies of *D. rosea* that have been sectioned, a situation conforming exactly to Julin's concept undoubtedly existed, for side by side with easily recognized young developing buds a number of metamorphosed larvae were unmistakably present. The limits of each larval unit were sharply shown by the clearness and firmness of the test substance of the young colonies compared with that of the parent colony in which they were embedded (Text-fig. 3). Further proof that they were what they seemed to be was furnished by the fact that all stages were found from intact but trapped tadpole larvae to forms in which not only was metamorphosis completed, but the oozoid itself resorbed. In the last and most advanced cases, the oozoid was represented by surviving traces of its original ampullae together with the persisting pigmented otolith and ocellus (Text-fig. 3B). The first blastozooid was present in each, together with one other abortive bud of the same generation, while no others were discernible. Therefore, tadpoles may inadvertently become retained in the parent colony, and if so, may undergo metamorphosis and give rise to a first blastozooid generation upon dissolution of the oozoid. It is to be noted that only one of the original four or five buds actually develops, presumably the largest, and that the others fail to develop and even disappear under conditions clearly optimal for their development.

Distaplia bermudensis

The foregoing account of *D. rosea* is to some extent introductory, but has been emphasized since it comprises the bulk of the observations that have been made upon living material. Colonies of *D. rosea*, however, are tiny, rather difficult to obtain, and hardly afford an adequate material basis for a general study. Colonies of *D. bermudensis* may also be rather small, as, for example, most colonies found in the Bermudas proper, but along the shores of the Gulf of Mexico colonies of a *Distaplia* that appear to be the same species may attain relatively enormous sizes, and it is this type, collected at Englewood, Florida, that has been used in the following account.

Structure of zooid

A large active zooid is shown in Text-fig. 4A, drawn to the same scale as the oozoid and first blastozooid of *D. rosea* in Text-fig. 2. As in these, the branchial sac has four rows of dorso-ventrally elongated stigmata on each side. The gut loop is differentiated into an oesophagus, stomach, post-stomach or mid-intestine, and a long rectal ascending limb opening anteriorly into the atrial cavity. The gonads lie in the loop of the intestine, and both sperm duct and oviduct follow the ascending limb of the intestine to open beside the anus. Extending into the depths of the common test of the colony from the posterior end of the zooid are a pair of closely applied vascular tubes. They communicate with one another usually in two regions, often near their distal ends,

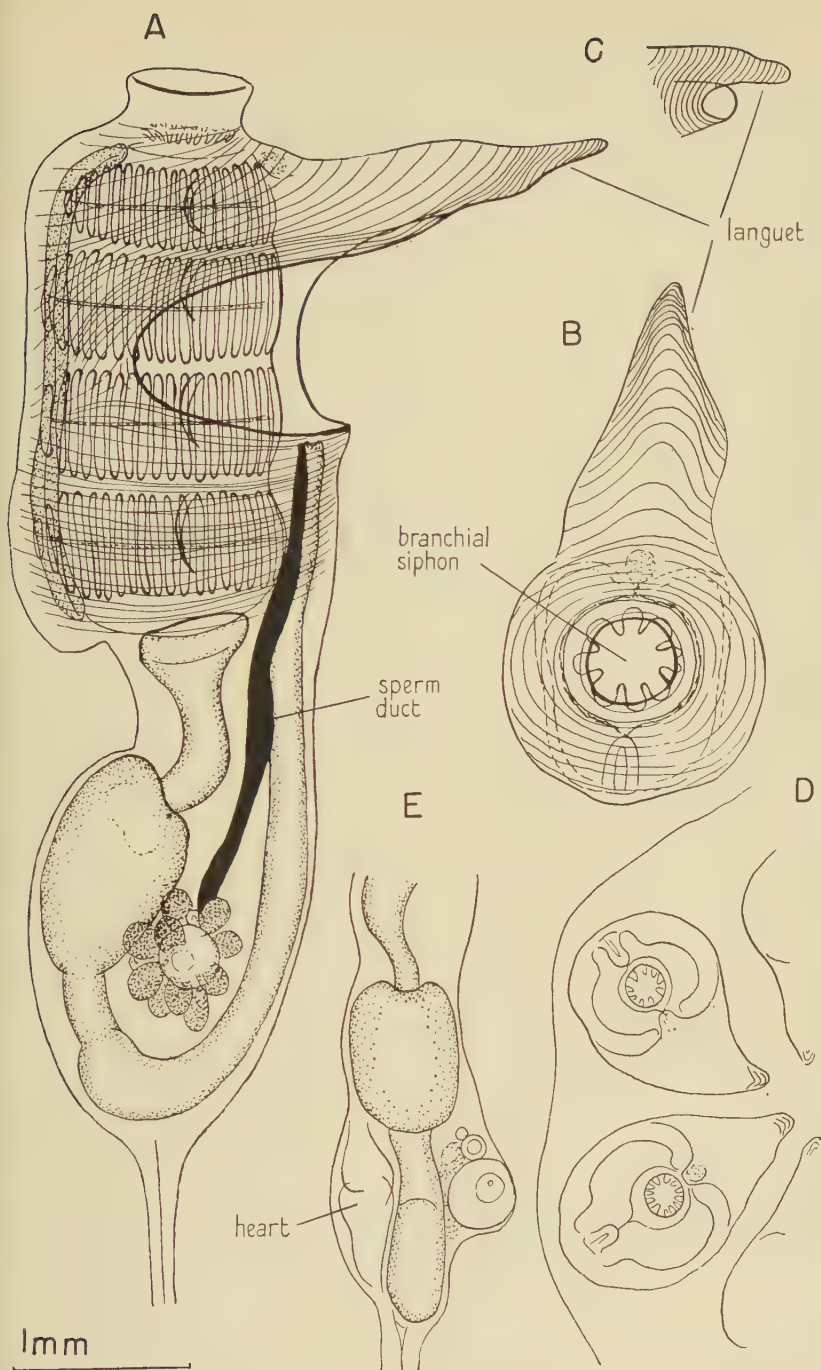
and usually also a relatively short distance from the body of the zooid at junction marked by ampullary swellings (Text-fig. 5C). There is little doubt that these swellings are homologous with the epidermal ampullae already seen in the first blastozooid and the oozooid. In the zooid of *Distaplia bermudensis* illustrated in Text-fig. 4A the branchial siphon is simple and circular, but the atrial siphon is greatly hypertrophied, though more as an aperture than a siphon.

The enlargement has a double effect. The aperture itself extends posteriorly to the level of the lowest row of stigmata, and ventrally, on one side, it cuts back almost to the endostyle, thereby exposing a large part of the inner branchial sac. The upper or anterior margin of the siphon, in place of being cut back like the lateral margins, extends out dorsally from the thorax in the form of a large and long tapering languet (Text-fig. 4B). This is in marked contrast to the condition in the oozooid and first blastozooid of *D. rosea* (and there is evidence that the oozooid of *D. bermudensis* is similar), in which the atrial siphon is small and circular like the branchial siphon. Smaller zooids from the same colony, however, possessed an atrial siphon like that shown in Text-fig. 4C, with the upper margin of the siphon just starting to protrude. The presence and shape of the languet is correlated with the presence and form of zooid systems in mature colonies. Looking at the upper surface of a colony the zooids are seen to be arranged in more or less star-shaped systems of about six zooids each. All the languets of one system of zooids point to the centre of the system and together form the margin of a common cloacal aperture (Text-fig. 4D).

The heart and gonads lie on opposite sides of the gut loop below the stomach (Text-fig. 4E). The lower end of the heart opens into one of the two vascular epidermal tubes already mentioned. The other tube opens into lacunae towards the stomach and gonads.

The zooid shown in Text-fig. 4A is clearly a sexually mature form with a rosette of testicular follicles and a sperm duct congested with sperm. Growing ova of various sizes lie at the centre of the male follicles. The sexual maturity of the zooid, however, refers only to the male component, and zooids of this species attain male and female phases at different times. Large zooids, when newly functioning, have fully matured testes and full sperm duct. Only as growth proceeds do they acquire mature ovaries and oviduct.

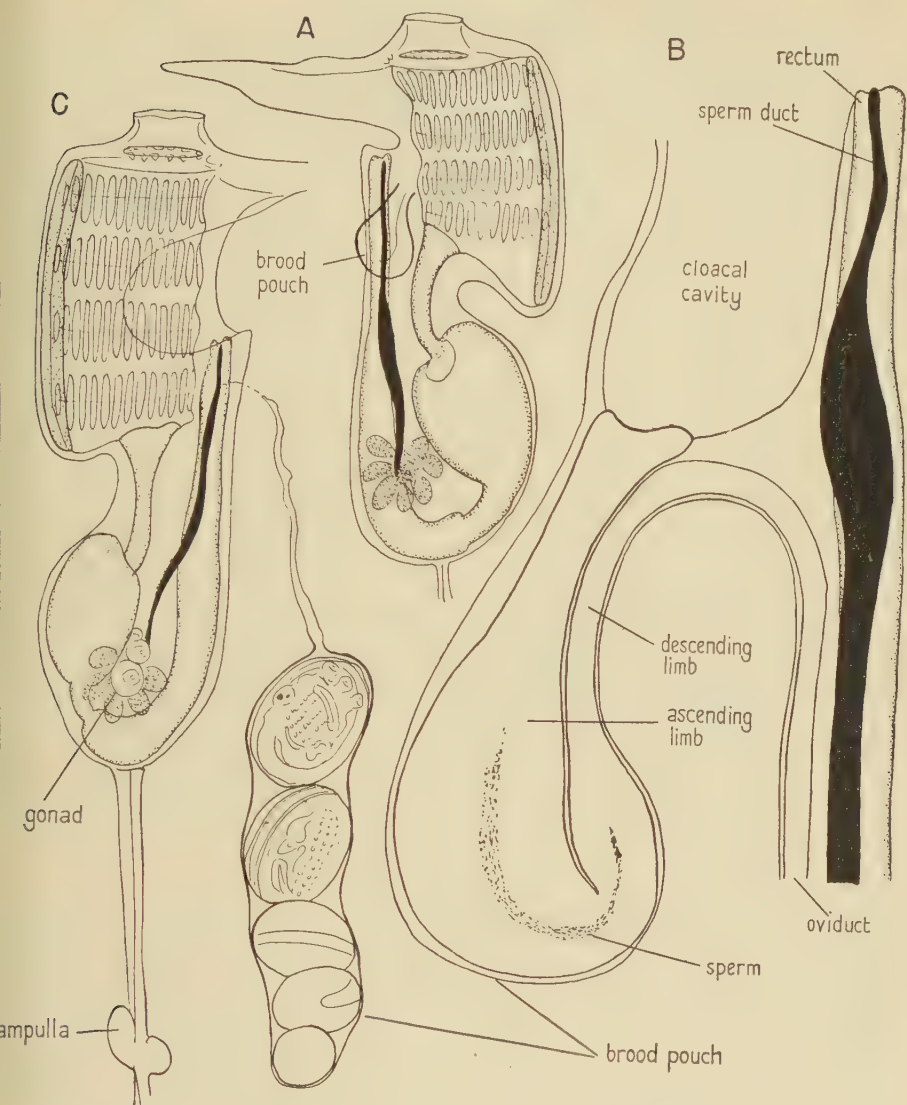
Following a rather small but appreciable amount of growth, the largest ovum attains its final and maximum size. Coinciding with this the brood-pouch appears for the first time as a small pear-shaped protrusion of the body-wall near the base of the dorsal atrial cavity (Text-fig. 5A). The nature of the brood-pouch was examined by Bancroft (1899), who first figured it in its early stage of development. He recognized that the epidermal sac contained an inner tube doubled upon itself and continuous at one end with the oviduct and at the other with the atrial cavity, but was unable to determine to what extent oviduct and atrium each contributed to the pouch. He was, however,



TEXT-FIG. 4. *D. bermudensis*. A. Mature zooid in primary, male, phase, showing enormous gape of the atrial siphon and the growth of the dorsal rim as an atrial languet. B. Dorsal view of branchial siphon and atrial languet. C. Atrial siphon from younger zooid at onset of local atrial growth. D. Part of a zooid system to show convergence of atrial processes toward common cloacal aperture. E. Ventral view of abdomen showing heart and gonads on opposite sides of the intestinal loop.

able to account for the fact that advanced brood-pouches contained embryos arranged so that the youngest was at the bottom and the oldest at the top. He saw accurately (in *Distaplia occidentalis*) that eggs pass up the oviduct and down a narrow descending duct to the base of the pouch, subsequent eggs pushing the first ones up the ascending part of the double tube. This account is fully confirmed for the present species. The condition, however, can be further clarified, and is illustrated in Text-fig. 5B. The sperm duct is not involved in the least in the formation of the brood-pouch, but persists as a duct closely applied to the rectal intestine and opens alongside the anus into the atrial cavity. The oviduct, on the other hand, grows distally in such a way that the distal end remains opening into the base of the atrial cavity while immediately posterior to this it bulges out as a loop, adjacent epidermis accommodating to it. The loop consists of a narrow descending limb of a diameter no wider than the unmodified oviduct, and a much wider ascending limb. Long before the first egg passes up the oviduct proper and into the pouch, considerable masses of spermatozoa can be seen as a diffuse column within the pouch, having entered through the atrial oviducal opening (Text-fig. 5B). Eggs subsequently pass up the oviduct and down the descending limb in the pouch. In so doing they become compressed and sausage-shaped, regaining the typical spherical state upon emergence into the base of the wide ascending limb. Each egg passes up and enters the bottom of the pouch in this manner as maturation is attained, and a series of perfectly graded embryos from early cleavage to mature tadpole stage are usually found in well-developed pouches. The pouch continues to grow as the eggs successively reach it, until a characteristic maximum length is reached. Finally, even though there may still be growing ova in the ovary, the neck of the pouch becomes greatly attenuated and constricts off from the body of the parent (Text-fig. 5C). The elongated brood-pouch thus becomes isolated from the zooid and in fact persists with its contained series of developing embryos usually long after the dissolution of the zooid that originally produced it.

The isolated and abandoned brood-pouches with their embryos and tadpoles are said to migrate through the matrix of the colony to liberate the larvae as they become ready. A mechanism for such a migration is difficult to imagine, and in fact there is no migration in the sense implied. The same result is reached by a progressive sloughing off of the surface test material of the colony until the anterior ends of the pouches come to lie at the surface. This is an inevitable result of the dissolution of the parent zooids. The test matrix is a complex medium and is one that needs to be actively maintained by adjacent living tissues. With the reduction of the zooids, the superficial layers of test soften and degenerate, and the colony surface is in effect cut back to the level of the pouches. While sloughing at the surface does bring the pouches close to that region, some migration is not excluded, for a mechanism does not need to be understood in order to exist. In the case of buds arising from some source deep within the colony, migration towards the surface and orientation there to form systems undoubtedly occurs.



TEXT-FIG. 5. *D. bermudensis*. A. Mature zooid at onset of female or hermaphrodite phase with brood-pouch at early developmental stage. B. Enlarged view of young brood-pouch to show its origin as a bulging local extension of the distal oviduct. C. Mature zooid in final phase with brood-pouch filled with graded series of developing embryos, the neck or stalk of the pouch constricting off from parent at base.

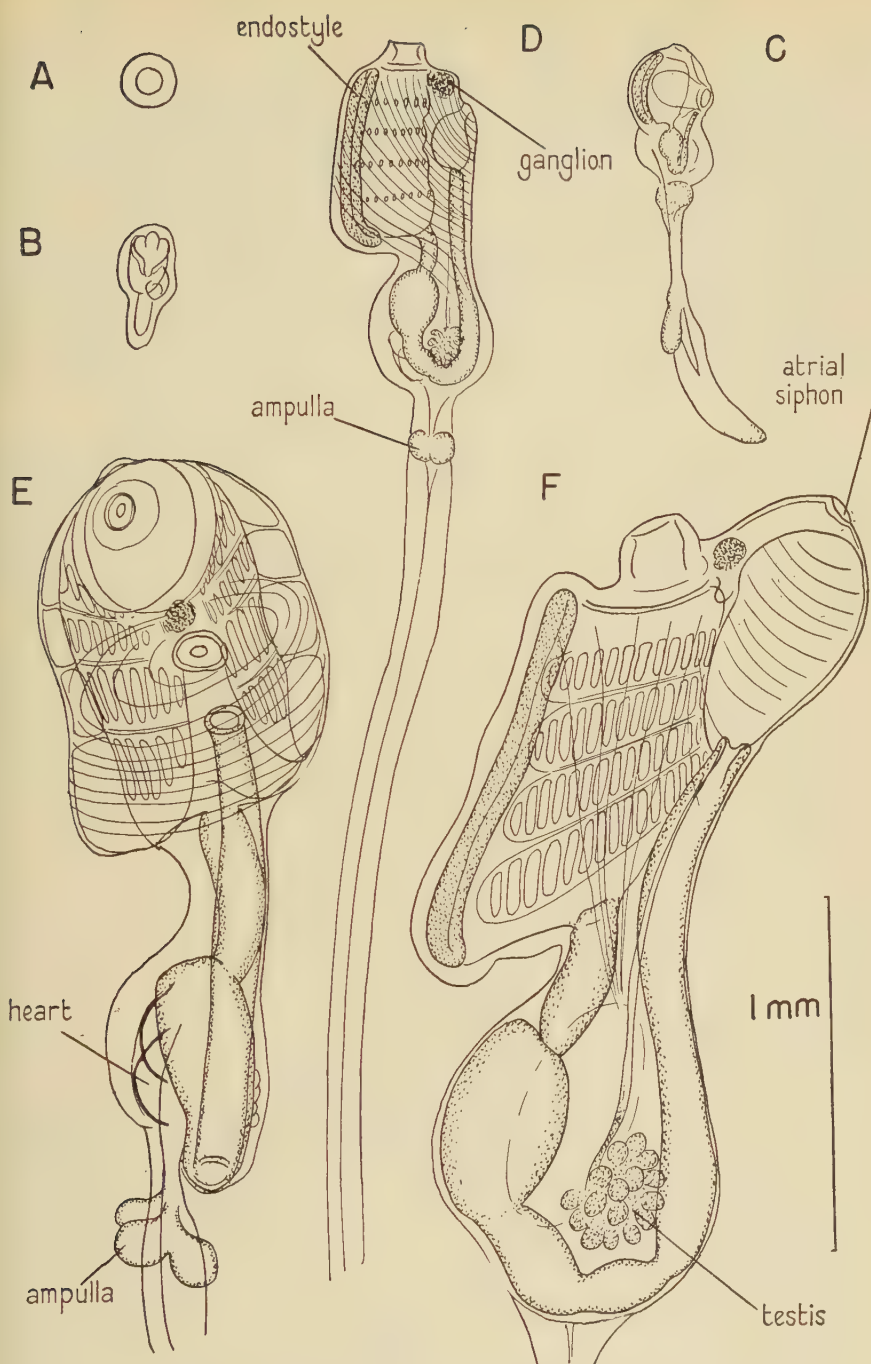
Growth of Buds

The gross development and growth of the buds is described here in order to complete the growth-cycle of the zooid, leaving the question of early morphogenesis until after the problem of bud origins has been discussed.

Text-fig. 6 shows the growth of the bud from the time shortly after its origin, when it consists essentially of a spherical envelope of epidermis enclosing a more or less spherical inner mass of cells, until shortly before it attains functional activity with siphons opening to the surface (Text-fig. 6E and F). In a quantitative sense, the amount of growth is immense and raises the question of nutrition. The bud from the start is an isolated unit within the matrix of the colony and obviously possesses but a fraction of the substance contained in the developed zooid. The only possible source of nourishment is the surrounding more or less fluid organic medium. There is no accumulation of reserve cells within the bud, no migration of food-laden cells into the bud system during its development, and no connexions made subsequently between the closed bud system and that of any more developed zooids. In any well-established colony degenerated zooids or their dissolution products are seen together with functional zooids, and a rich growth medium undoubtedly exists. That such is the source is further shown by the fact that the first blastozooid of *D. rosea* remains virtually in a state of arrested development all through the functional period of the oozooid, undergoing a rapid growth simultaneously with the dissolution of the oozooid and the consequent availability of its disintegration products.

The primary bud sphere elongates from the first and, at the stage and size shown in Text-fig. 6B, possesses the basic organization of the zooid, with branchial and atrial divisions, endostyle, neural complex, heart, digestive loop, and epicardium. Further development is mainly an elaboration of these parts. The four rows of stigmata are imperforate but are discernible in the stage of Text-fig. 6C. At this stage the abdominal epidermis has extended posteriorly as a long tubular outgrowth with a tendency to form terminal ampullae, anastomoses, and ampullary swellings close to the base of the abdomen proper.

In the next stage (Text-fig. 6D) the stigmata have perforated, and the heart is already beating even though the zooid as a whole is far from a functional state or size. Three features are outstanding. There is an enormous elongation of the pair of posterior vascular epidermal vessels, an elongation which in itself tends to drive the zooid up towards the colony surface, and a sharper differentiation of the cross-connecting ampullae. The gonad is clearly visible as a relatively large mass situated within the intestinal loop. Both branchial and atrial siphons are clearly formed though not open, and the muscle bands of the body-wall are readily seen. At this stage the atrial siphon has a small circular aperture exactly like that of the branchial siphon. In the final stage, shown in dorsal and lateral view in Text-fig. 6E and F, there is a much larger size but little structure change. The gonad is more clearly seen to be testis only, while, right up to functional activity at least, the atrial siphon remains small and circular, with no resemblance to the wide-gaping siphon of the mature zooid with its long languet. The posterior growth of the vascular vessels is due, for the most part at least, to continuous terminal proliferation of the distal extremities, and is doubtfully an example of true relative growth.



TEXT-FIG. 6. Gross development of a bud (*D. bermudensis*). A. Bud at initial size and form. B. Bud with essential morphogenesis complete. C. Stage with stigmata represented at 4 imperforate bands. D. Stigmata well perforated, testes well developed, and vascular stolon enormously elongated. E, F. Dorsal and lateral views of advanced stage, showing small circular atrial siphon.

With regard to the rest of the developing zooid, there appears to be little significant change in body proportions between the stages shown in Text-fig. 6B and F; in other words, during this phase and excluding the vascular stolon, growth of the whole seems to be a unitary process with all organs and tissues growing at the same pace.

With the attainment of functional activity by the zooid the siphon begins to exhibit relative growth culminating in the form of siphon already described for the mature adult. It is as definite an example of relative growth as any, and implies a sharp change in the developmental system at the time of zooid functioning, from a closed morphogenetic system to an open one.

Gonad formation in D. bermudensis, D. rosea, and D. magnilarva

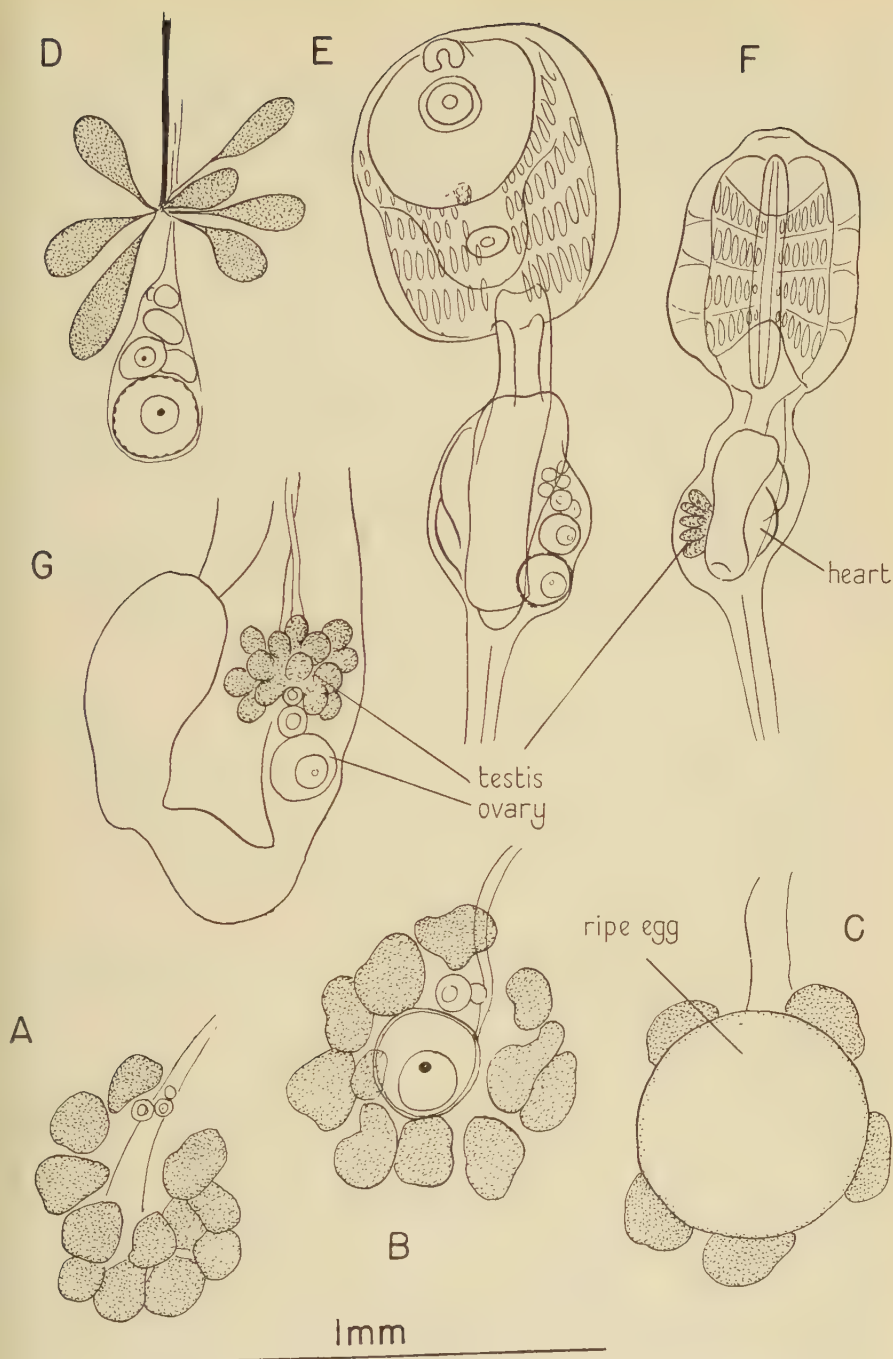
Leaving aside for the present the origin and nature of the gonad Anlage, the gonad as initially discernible in whole mounts develops in the same manner as the rest of the zooid, namely, as a growing mass of reproductive tissue exhibiting no further change except for size throughout the developmental period up to the functioning of the zooid, after which relative changes occur.

The condition in zooids of *D. bermudensis* at the end of bud development is shown in Text-fig. 7A, and this is the condition of the gonad even in the youngest stages. It grows without change until the zooid as a whole becomes functional, after which the largest ovum rapidly grows to its maximum (Text-fig. 7A, B, C), successively followed by several others. While clearly hermaphrodite, the zooids are at first functional males and only later function as females, so that finally only female structure may be visible.

In *D. rosea* the youngest buds are seen to contain gonads in which both male and female components are equally represented. The condition at the time of initial functioning of the zooid is shown in Text-fig. 7D, testicular follicles converging to the base of the sperm duct, while the ovary extends from the body-wall in a pear-shaped sac.

D. magnilarva is of greater interest. Uljanin (1885), studying live colonies at Naples, concluded that not only were all the zooids of any one colony all of the same sex, either male or female but never hermaphrodite, but that there was a regular alternation from one sex to the other. Caullery (1895) confirmed Uljanin that colonies were unisexual, but considered young buds (0.8 mm. long) to be hermaphrodites, changing into unisexual adult zooids (6.0 mm. long) through a regression of one sexual component on the other at a relatively early stage, whichever happened to be the smaller.

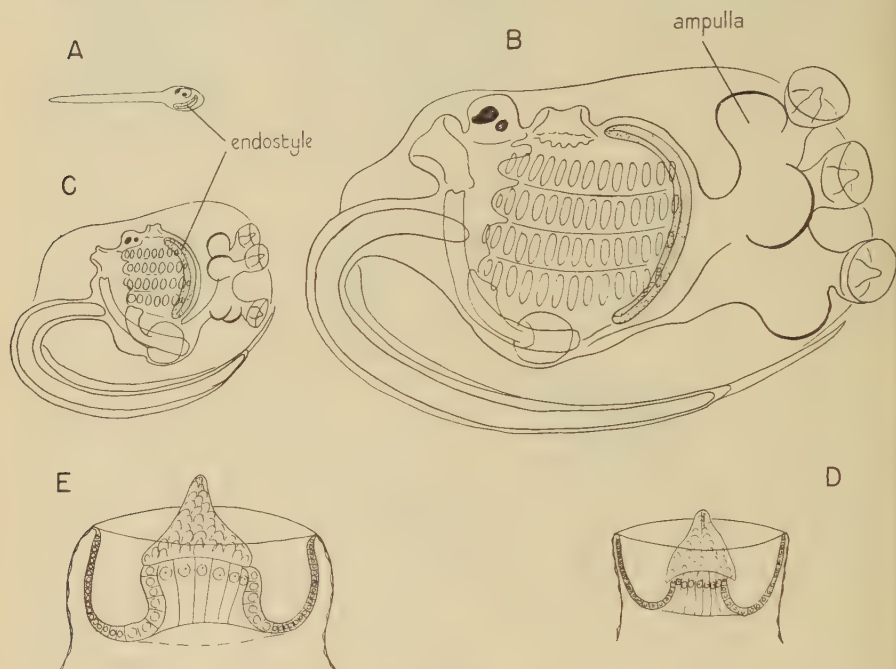
In the only colony of *D. magnilarva* examined here, very large and obtained from Naples, the great majority of the zooids were purely female with large ovaries and brood-pouches with many embryos. Very young buds (0.7 mm.) in the vicinity of mature female zooids showed only developing ova (Text-fig. 7E). In one region of the colony, however, about two dozen zooids had no brood-pouches, no sign whatever of ovaries, but large well-developed rosettes of testis. In their vicinity the smallest buds appeared to possess only



TEXT-FIG. 7. Gonad development in various species of *Distaplia*. A, B, C. Stages in gonad development of *D. bermudensis* from initial functioning of mature zooid to formation of first ripe ovum. D. Gonad of *D. rosea* in developing bud. E, F. Young buds of *D. magnilarva* showing female and male types respectively. G. Occasional hermaphrodite gonad in young *D. magnilarva* bud.

developing testes (Text-fig. 7F). In a few young buds in different parts of the colony, however, hermaphrodite gonads (Text-fig. 7G) were encountered.

These conditions raise interesting questions concerning the general mechanisms of sex determination, and will be further discussed following an analysis of the origin and nature of gonad Anlagen in buds too small to be



TEXT-FIG. 8. Size and organization in *Distaplia* tadpoles. A. Tadpole of *Ascidia* for scale comparison. B. Tadpole of *D. magnilarva*. C. Tadpole of *D. rosea*. D, E. Adhesive organs of *D. magnilarva* and *D. rosea* drawn to same enlarged scale, showing some cell numbers.

studied except by serial section. Functional unisexualism is found elsewhere among ascidians only in species of *Colella*, a genus closely related to *Distaplia*.

PART II. THE DEVELOPMENT AND NATURE OF THE TADPOLE LARVA

The embryonic development of *Distaplia* has been described at length by Dawidoff (1889), but at a time when little or nothing was known concerning the distinctive cleavage patterns of the ascidian egg in other forms, and when the main interest lay in the general chordate character of development and its relation to the ascidian organization subsequently formed. The nature of the tadpole as a functioning organism was neglected, while the pattern of early cleavage was overlooked. The present account is therefore mainly of these two phases and does not emphasize the intervening span of development to which Dawidoff gave adequate attention.

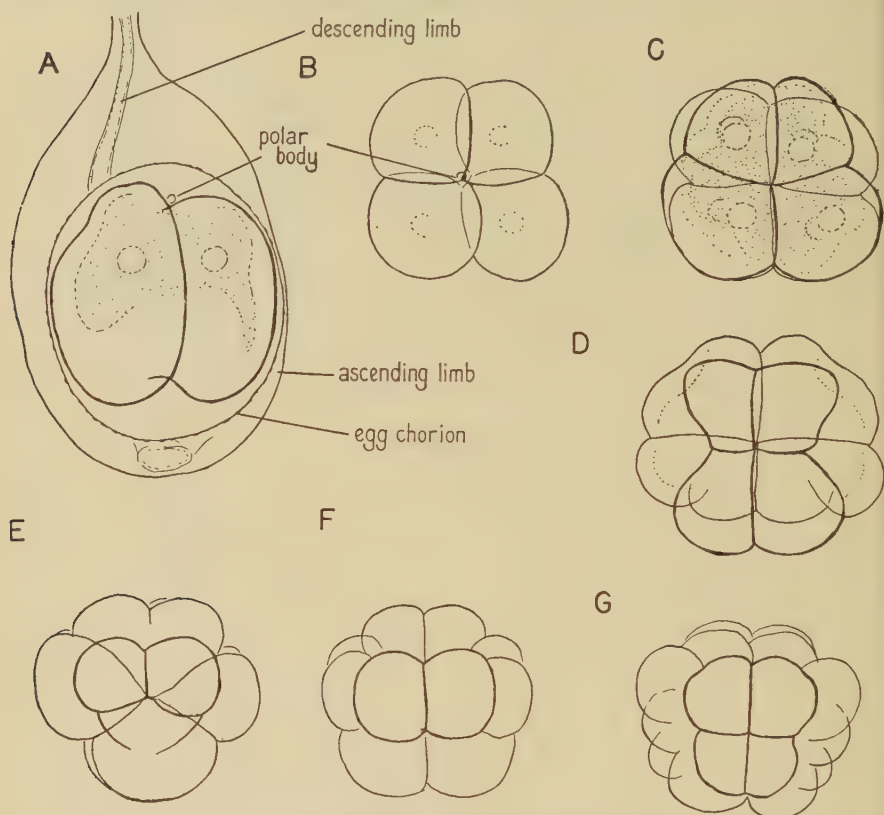
As an introduction to the nature of the tadpole, Text-fig. 8 illustrates the general form and structure, and particularly comparative sizes. The smallest and largest tadpoles of *Distaplia* species are shown, those of *D. rosea* and *D. magnilarva*. Those of the other species studied, namely, *D. bermudensis*, *D. garstangi*, *D. clavata*, and *D. occidentalis*, are intermediate in size between these two extremes. A tadpole of *Ascidia* is shown at the same scale. It is typical in size for all oviparously developing ascidians, whatever the family. It is clear, however, that variation in the size of the *Distaplia* tadpoles does not significantly affect their organization. The relative shortness of the tail of *D. magnilarva* tadpoles is in part due to the fact that the tail is composed of comparatively solid tissue, whereas the trunk and anterior structures consist virtually of 2-dimensional sheets of tissue, and a difference in egg diameter therefore expresses itself more strikingly in the trunk than the tail. The sensory organs differ in size but not in number or type of cellular components, while in the case of the adhesive organs (Text-fig. 8D, E) the size of the cells varies but not the number or organization. In contrast, however, multiple structures of the permanent ascidian organization, i.e. gill slits, do vary in number approximately with the size of the whole, the constituent cells varying in number but not size.

Cleavage

In most species of *Distaplia*, eggs in process of early cleavage are rarely encountered and are in any case difficult to orientate, the more so when they lie somewhat congested at the bottom of a brood-pouch filled with developing embryos at all stages. The present account is based on colonies of *D. clavata* collected in July by M. J. Dunbar at Ungava Bay. They are peculiar in that all are almost at the same stage, with young brood-pouches containing either one or two eggs in the earliest stages of cleavage, and, from these abundant examples, the patterns could be readily observed; they are illustrated in Text-fig. 9.

In Text-fig. 9A a complete young pouch is shown with both limbs of the pouch indicated. The contained egg, in the 2-cell stage, is enclosed in a chorion typical of viviparous forms with follicle cells flattened out over its surface. Both of the first two cleavages pass through the animal pole as indicated by the position of the polar body. The third cleavage is equatorial, and, in spite of the large size and comparative yolkeness of the egg, produces a bilaterally arranged group of 4 on 4 cells as described for the small egg of *Styela* by Conklin (1905). This stage and its imminent transition to the 16-cell stage is shown in Text-fig. 9C and D. The 16-cell stage proper is shown from lateral, posterior, and dorsal sides in Text-fig. 9E, F, and G respectively. The characteristic bilateral pattern is as clearly marked, therefore, in the developing egg of *Distaplia* as it is in any other ascidians. Scott (1946) has shown that the comparably yolky egg of *Amaroucium* is essentially the same, and it follows accordingly that increase in size and yolk content of ascidian eggs in no way significantly affects the course of early development, and the basic developmental patterns persist.

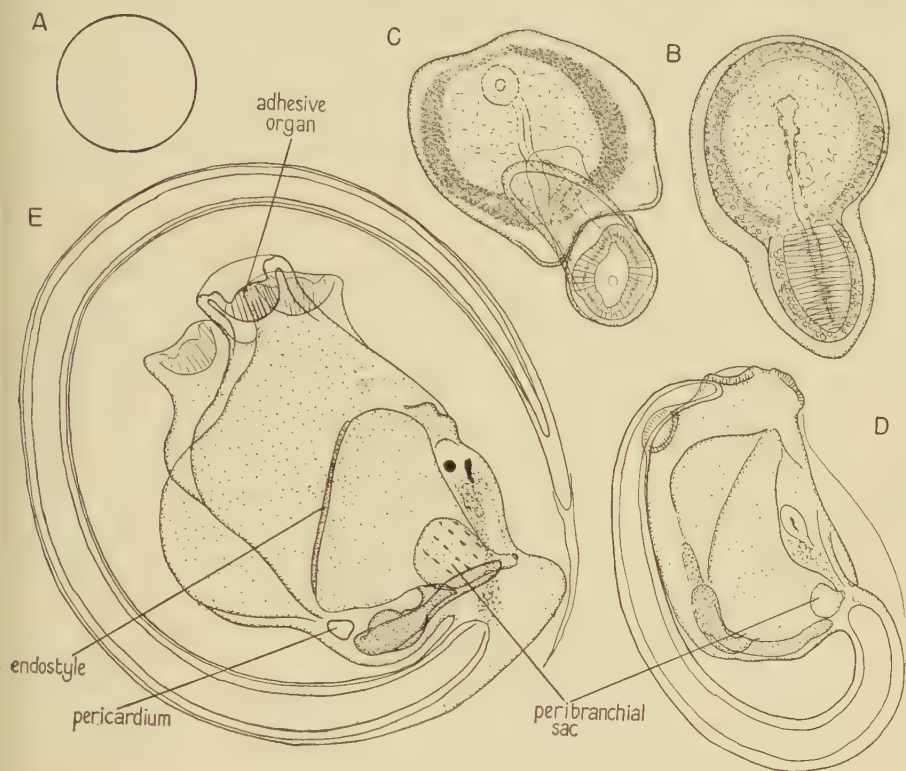
One of the more striking features of later development is the great increase in size from the egg to the tadpole (Text-figs. 10–12, especially fig. 10A–E). While these illustrations are of *D. bermudensis*, the expansion is typical of all species of the genus and is mainly a reflection of the large amount of yolk within the egg. Text-fig. 10B and C represent young embryos showing two



TEXT-FIG. 9. Cleavage stages of *D. clavata*. A. 2-cell stage within brood-pouch. B. 4-cell stage. C. 8-cell stage. D. 8-cell dividing to 16-cell. E, F, G. Three aspects of 16-cell stage.

stages in the formation of the notochord. Approximately 40 notochord cells are produced, as in all other ascidians, and the tail grows out under the pressure of the swelling cells, at first as two interdigitating rows (Text-fig. 10B) and then as a single column of 40 (Text-fig. 10C). In this last stage the tail is shown bent over so that the tail muscle-cells are seen in optical section around the notochord. These are also now present in the full final number. Mesenchyme cells also appear in large numbers, between the epidermis and endoderm. Succeeding stages show a rapid overall expansion, the epidermis and endodermal envelopes becoming widely separated from one another and the tail with its enclosed notochord extending around the full circle of the

enlarging trunk (Text-fig. 10D and E). In the stage shown in Text-fig. 10D, the larval sensory vesicle exhibits the first sign of pigmentation of the sensory cells forming the ocellus and otolith, while the three anterior adhesive organs



TEXT-FIG. 10. Development of the embryo (*D. bermudensis*), to same scale as tadpole stages in Text-figs. 11 and 12. A. Egg outline for size comparison. B. Embryo with trunk and tail differentiated, full number of notochord cells delimited. C. Somewhat older embryo with branchial siphon forming, and tail recurved showing attainment of full number of tail muscle-cells in optical section. D. Young tadpole form with first trace of sensory pigment, peribranchial sac, and rudiments of adhesive organs. E. Later stage, with stigmata appearing and pericardium formed.

are evident as shallow cups of thickened epidermis. At the same time the peribranchial sacs appear as a pair of dorso-lateral invaginations.

The later stage (Text-fig. 10E) shows all developmental units already present, with four rows of dividing stigmata visible in the now-conjoined peribranchial sacs, a digestive loop with oesophageal, stomach, and rectal divisions, a completely segregated heart, distinguishable endostyle, &c. The adhesive organs each are differentiated into a central mass of columnar cells and a marginal cup wall giving a sucker appearance to the whole.

The two stages illustrated in Text-fig. 11 are even more recognizable as tadpoles, although they do not represent the active free-swimming stage. The

main interest in these stages, taken together with the final active stage (Text-fig. 12), lies less in the continued differentiation of structures and organs than in the relative growth or form changes occurring in the anterior trunk, the branchial wall, and the distal abdominal region. While each progressively changing process is a phenomenon apparently unrelated to the others, there is equally a rigid correlation in a time sense, so that a knowledge of the stage reached in any one case accurately indicates the state attained by the other two. Since these are, however, essentially growth activities, each is best described in terms of the three stages illustrated without much reference to the stage as such.

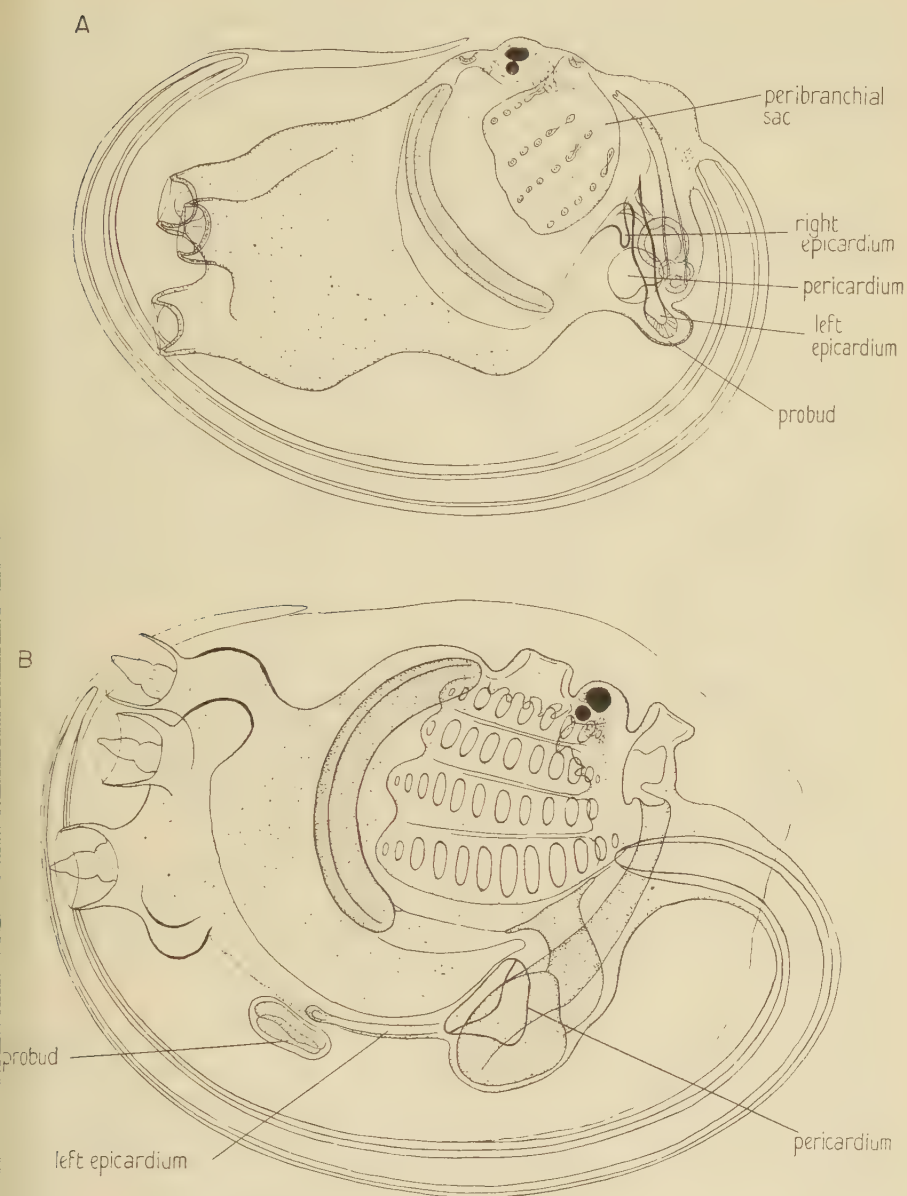
Branchial Wall

The branchial wall is essentially the area of the combined peribranchial and endodermal layers. The peribranchial sacs are ectodermal invaginations on both sides, almost at once fusing mid-dorsally to form the atrial siphon, and coming to lie with the inner side of the sac in contact with the outer surface of the endodermal pharynx. For a while the rapidly expanding sacs appear to slide over the endodermal wall, but this may not be actually the case. When the sac has spread about one-third the way across the endodermal wall toward the endostyle, four rows of thickened tissue appear, the four rows of stigmata, at first imperforate but quickly breaking up into separate perforate units. As the peribranchial sac appears to spread toward the endostyle anteriorly, and also dorso-ventrally, the stigmata increase in number to about twice that originally recognizable, until each row consists of about a dozen well-formed gill slits (Text-figs. 11 and 12). The reason for doubting that the peribranchial wall is sliding along the endodermal wall towards the endostyle is that the stigmata are perforate at a relatively early stage in the process, and as perforate structures clearly undergo elongation and division. Since a perforate stigma involves a fusion and perforation of both peribranchial and branchial layers, one of the two layers cannot really be extending relative to the other, and a more reasonable interpretation is that the combined area is growing and differentiating as a unit, while the purely endodermal wall between it and the endostyle becomes correspondingly reduced in some way.

The number of gill slits per row in the tadpole of any species of *Distaplia* is approximately half the number characteristic of the mature adult zooid of each species respectively.

Anterior Trunk

As seen in Text-fig. 11A, the anterior half of the trunk is wide and voluminous, with an extensive blastocoelic space within. In the later stage (Text-fig. 11B), four epidermal thickenings associated topographically with the three adhesive organs appear, while a deepening constriction begins on the dorsal side between these structures and the branchial region. This constriction progresses until it effectively cuts back in front of and below the branchial sac, so that the three adhesive organs and four ampullae are in fact connected

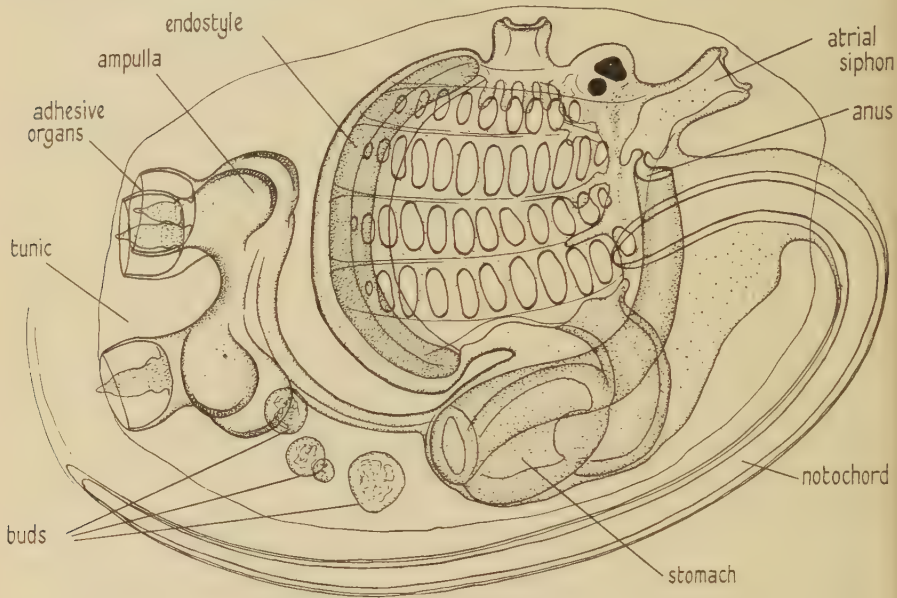


TEXT-FIG. 11. A. Stage with perforating stigmata, showing short right epicardium terminating above the pericardium, and left epicardium protruding ventrally to form the probud. B. Nearly mature tadpole with probud separated and elongating.

with the distal end of the abdomen by a pair of epidermal tubes or vessels (Text-fig. 12). The adhesive organs are transitory organs of the tadpole, but the ampullae and the vessels survive metamorphosis and represent the stolonic vascular vessels and ampullae characteristic of all blastozooids and described in the first section of this paper.

Bud Formation

The details of bud production and their subsequent history is the subject of the last part of this investigation. In the present instance the interest lies more



TEXT-FIG. 12. Mature tadpole of *D. bermudensis*. Adhesive organs and ampullae connected to trunk by narrow vascular stolon, probud constricted into four definitive buds.

in the relative time at which certain of these events occur. In Text-fig. 10A the epicardium consists of two evaginations from the floor of the pharynx, one short and ending immediately above the pericardial vesicle, the other extending to the ventral abdominal epidermis. This region of the epidermis, together with the distal end of the epicardial tube, bulges out as relatively thickened epithelia from the general contour of the body-wall.

In the succeeding stage (Text-fig. 11B) the protruding region has constricted off and is further elongated as the probud, while the residual epicardial tip, still relatively thick but much narrowed, has extended far anteriorly.

In the final tadpole stage (Text-fig. 12) the probud is segmented into four definite buds, the epicardium meanwhile having withdrawn to its abdominal site.

Structure of the Active Tadpole

The fully formed tadpole is in a sense a triple organism, consisting of the transient structures of the tadpole as a purely larval organism, the relatively permanent organization of the oozoid, complete except for minor changes in orientation and attachment, and among the buds is the first blastozoid destined to replace the oozoid (Text-fig. 12).

The oozoid organization is virtually complete and within a very few hours of actual functioning. The siphons show some contractility, and the heart beats and exhibits the characteristic reversal of beat from the first, even in the free-swimming tadpole phase. The branchial sac with its four rows of large definitive stigmata, dorsal languets, and branchial tentacles, and the alimentary canal, both appear morphologically complete, though cilia are not active. Anteriorly the ampullae, which will extend from the basal end of the future erect and attached oozoid, are connected to the region of the heart by the double vascular stolon.

The purely tadpole organs consist of three units, the adhesive organs, the sensory vesicle, and the tail, all of which are destroyed during the process of metamorphosis. The free-swimming period is short at the best, rarely more than of 2 or 3 hours' duration, and the habitat of most species is such that there can be little opportunity for selectivity on the part of the tadpole.

Adhesive Organs

There are three adhesive organs, the typical but not invariable number for ascidian tadpoles, and they are arranged also characteristically with one median and ventral and two dorso-lateral. Each consists of a central adhesive cone of glandular cells secreting the adhesive cement through a narrow tubular core, and a sucker-like cup of non-glandular epidermis surrounding the glandular part. There is apparently no real sucker function, for the cups become comparatively shallow at the time of attachment and seem to serve mainly to confine the spreading viscous secretion of the central structure to an effective area. As in the tadpoles of *D. magnilarva* and *D. garstangi*, in those of *D. bermudensis* the central glandular column of each adhesive organ consists of 6 or 7 high columnar secretory cells.

Sensory Vesicle

The sensory vesicle forms an obvious protrusion between the branchial and atrial siphons. The general organization is shown in Text-fig. 13. The vesicle and its components vary in size in general proportion with the tadpole as a whole. Otherwise the structures are fairly typical, in kind and arrangement, of those of ascidian tadpoles in general.

A dorsal view of the area between the siphons is shown in Text-fig. 13A, with the sensory vesicle proper lying side by side with the neural gland; the neural ganglion of the oozoid is situated in the midline between the sensory organs on one side and the gland on the other. Text-fig. 13B is a lateral view of the same structures, showing the unicellular nature of the otolith, the

3 lens-cells partly embedded in the pigmented mass of the ocellus. The pigment consists of closely packed granules contained in about a dozen cells arranged in the form of a cup (shown in Text-fig. 13C, an ocellus with pigment partly bleached out).

The Tail. The tail as a whole is twisted through 90° relative to the axis of the trunk, so that the cuticular membrane is horizontal.

The notochord consists of approximately 40 cells, as it does in all ascidian tadpoles examined in this respect. The intracellular vacuoles, however, are so enlarged that they have fused end to end to form one long cylindrical tube, with the nuclei compressed and lying in the containing membrane (Text-fig. 13E).

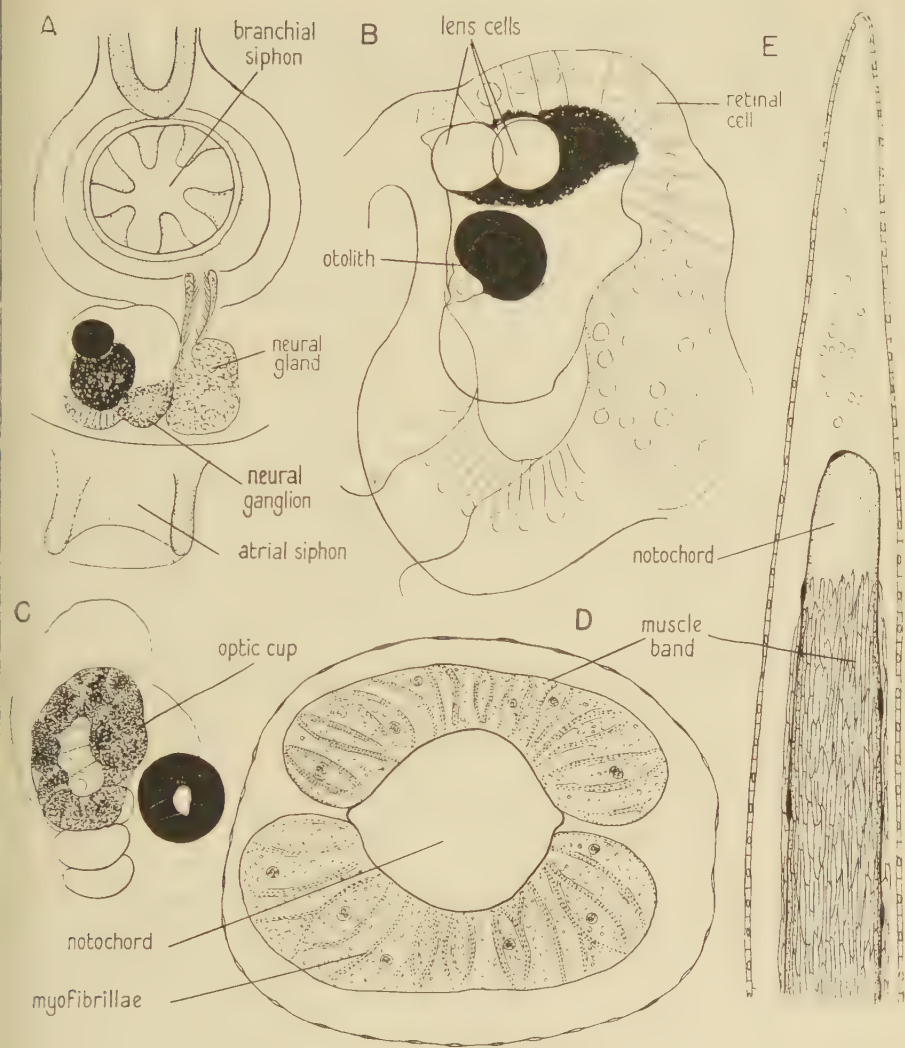
The notochord lies within a tapering epidermal tube of similar shape, but without coming into actual contact with the epidermis. This is especially evident at the tip, where the epidermal envelope is seen to extend a considerable distance beyond the distal end of the notochord.

The tail muscle-tissue consists of two bands, one above and one below the notochord (Text-fig. 13D). The bands adhere closely to the outer surface of the notochord, though they do not extend as far as its distal end (Text-fig. 13E), a condition again shared with most other tadpoles.

The muscle-cells are, however, unusual in two respects. In the tadpoles developing from all small eggs, the number of muscle-cells is as constant as the number of cells forming the notochord and is about 36. In the case of larger eggs, tadpoles developing from them may acquire tail-muscle tissue amounting to several hundred cells, e.g. *Amaroucium*, *Stolonica*. In *Distaplia bermudensis* the number slightly exceeds 2,000, several times as many as in any other form. The gearing of the development and differentiation of notochord and muscle-bands offers an intriguing comparative study. The second peculiarity is that whereas in most other tadpole types the fused cortical regions of the cells through which the continuous myofibrillae extend lie at the outer periphery of the bands, the fused cortical zones lie on radial axes from the centre of the notochord (Text-fig. 13D). The myofibrillae accordingly pass through the band of tissue only within the mass, and the surfaces that normally contain them are destitute. Whether the arrangement is a direct consequence of the extreme subdivision of the presumptive muscle-tissue and related shift in cell orientation or not, the effect is to produce a greatly extended area of cortical zone and therefore of contractile efficiency.

PART III. ORIGIN, FORMATION, AND EARLY DEVELOPMENT OF BUDS

All earlier accounts of bud formation in *Distaplia* concern bud production in the tadpole larva, as does part of this section. Efforts have been made previously to find direct evidence of formation of buds by adult zooids, all apparently without success. Julin (1896) concluded that none was formed and the buds seen in the larva gave rise to all subsequent generations of blastozooids. This has been supported by the fact that most observers have reported probuds in process of division in colonies of all ages. Daumezon



TEXT-FIG. 13. Details of tadpole structure (*D. bermudensis*). A. Dorsal view of sensory vesicle and atrial and branchial siphons. B. Reconstruction of sensory vesicle from adjacent sections showing unilocular otolith, lens cells, retinal cells, and ganglion. C. Dorsal view of partly bleached vesicle with otolith, 3 lens cells, and with centre of optic pigment showing sensory cones. D. Cross-section of tail with completely vacuolate notochord, and muscle-cells arranged with myofibrillae extending only through coextensive surfaces. E. Tip of tail showing relative extensions of muscle-cells, notochord, and epidermal envelope.

(1909), however, challenged Julin's conclusion on purely *a priori* grounds, though Brien (1939) has accepted Julin's opinion wholeheartedly, and in his excellent contribution on the budding and organogenesis of the blastozoid of *D. magnilarva* has limited his investigations to the larval stage, even though

living mature colonies were used to obtain the material. He refers to an earlier statement by Berrill (1935), that buds are produced by blastozooids as well as by larvae, as astonishing. On the assumption, therefore, that the only buds produced are those of the tadpole, Brien is forced into a description of certain related phenomena that appears to be a complete misinterpretation of his observations.

Accordingly the present account consists of a description of the manner in which buds are produced by blastozooids of every generation, a description and interpretation of the probuds formed by both larva and blastozooid, and finally a developmental analysis of the peculiar sexual conditions referred to in the first section.

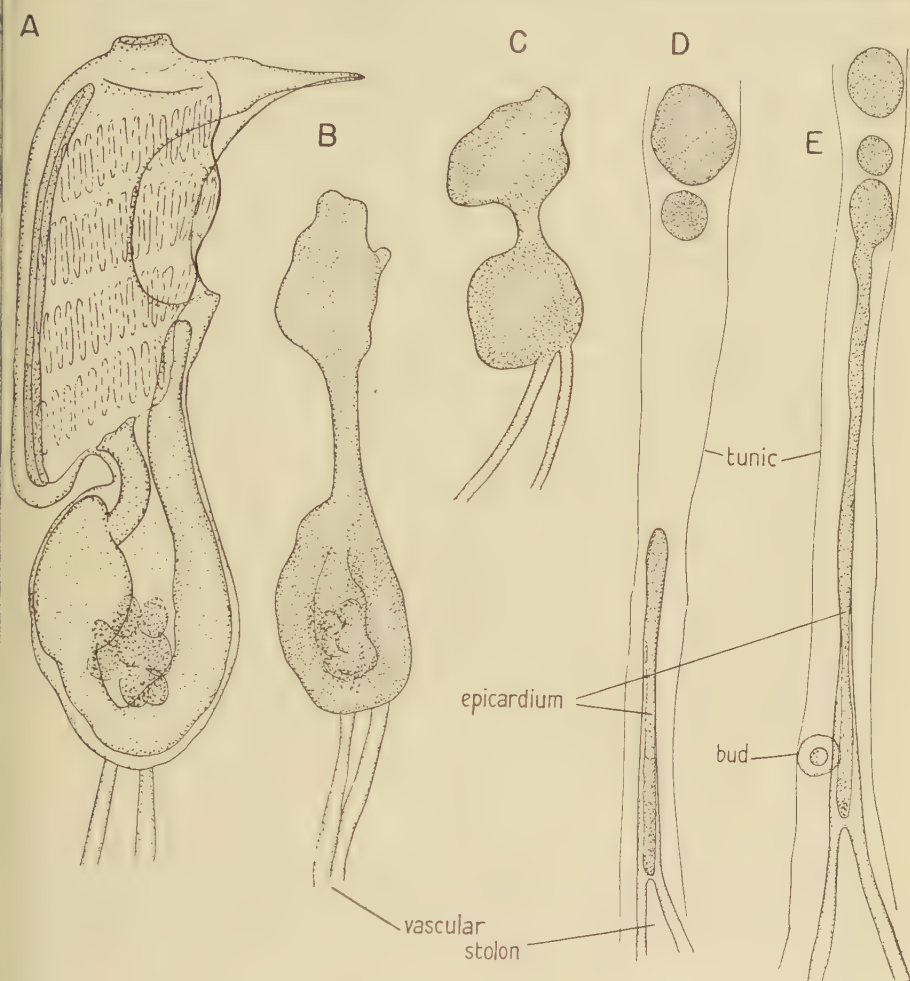
Origin of Buds from Blastozooids

The failure of previous investigations to demonstrate bud formation by the blastozooids of established colonies has been for the most part due to the nature of the colonies of the two species examined, namely, *D. magnilarva* and *D. rosea*. In the large colonies of *D. magnilarva* buds at all stages of development are generally to be found, and probuds in process of division usually encountered. It has been almost impossible to relate even the smallest buds with any adjacent structures, although Salfi (1925) suggested the vascular stolons as a source, an opinion rejected by Brien (1939). It should be emphasized that buds at their earliest recognizable stage are of a size comparable with the innumerable trophocytes scattered through the colony matrix, and that nutrition of the developing bud is maintained without contact with any other organized structure.

Colonies of *D. rosea* are comparatively small and each colony head consists usually of no more than a dozen zooids. Many such colonies have been sectioned entirely and it is possible to correlate the stages attained by co-existing generations. These observations may be summarized as follows. Very young buds, not probuds, are not invariably present. In most cases only developing buds with a recognizable degree of morphogenesis are to be seen coinciding with the presence of functional zooids. Primary buds are present in numbers only in colonies in which the adult zooids are degenerate except for persisting brood-sacs with embryos. If buds are produced by blastozooids, the evidence suggests an origin at the end of the individual blastozooid growth cycle. Likewise the segregative process must be of brief duration or buds in actual formation should have been seen.

With these tentative conclusions in mind, the large colonies of *D. bermudensis* were cut into small pieces, each piece including zooids near the surface and the matrix below them for the full thickness of the colony. In any one region zooids were found to be more or less at the same stage, newly functional with testis alone well developed, larger individuals with brood-pouches, or undergoing regression. Each stage was examined, from young developing buds through all functional stages up to and including the regressing zooids as long as anything could be recognized as having been a zooid.

No evidence of bud formation was observed in any of the developing or active stages. When it came to the study of regressing zooids the results were somewhat unexpected. Judging by the comparatively small number of zooids



TEXT-FIG. 14. A, B, C. Stages in degeneration of mature zooid of *D. bermudensis*. D. All but final stage, with zooid reduced to minimum but epicardium surviving and extending. E. Similar stage to D, with bud formed near posterior tip of epicardium and lying within stolon tunic limits. .

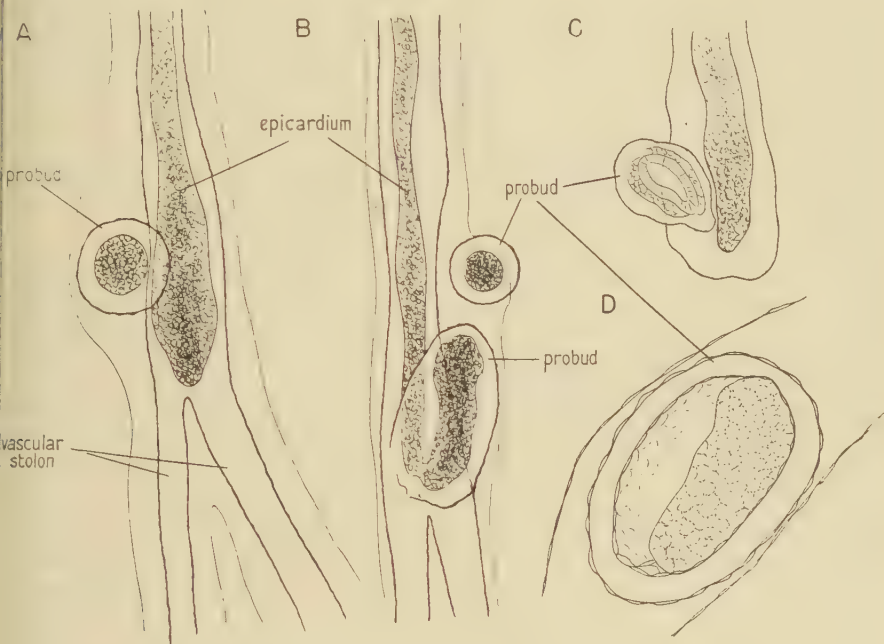
found undergoing regression, it is probable that the whole process is relatively brief compared with the growth cycle as a whole. Text-fig. 14A, B, and C show three early stages of regression, drawn to the same scale as the mature zooids illustrated in Text-fig. 4. The histological details of regression are not relevant here, and have been well worked out by Caullery (1895). In effect, however, the whole differentiated structure of the zooid progressively contracts as tissues shrink, cells autolyse, and phagocytosis ensues, usually

leaving finally one or two small residual masses (Text-fig. 14D), surrounded by heavily food-laden trophocytes. The important fact is that the dissolution process involves the thorax and abdomen but permits the survival of posterior structures. If the regression stages shown in Text-fig. 14C and D are compared, three features become evident. There is continued regression of the thorax and abdomen. There is survival, on the other hand, of the pair of proximal vascular epidermal vessels, while part of the original epidermal wall of the abdomen, together with all or part of the epicardium, not only survives but becomes markedly lengthened. This extension is much greater in the somewhat later stage shown in Text-fig. 14E, and consists of the region occupied by the epicardium, so that the crotch of the vascular processes becomes pushed progressively posteriorly. A comparable extension of the abdomen or post-abdomen, as the case may be, is characteristic of such synoicids as *Aplidium*, *Amaroucium*, and *Morchellium*, differing mainly in the survival in these forms of most of the contained structures and organs. In the last stage (Text-fig. 14E) a small bud is visible near the posterior tip of the epicardium, situated within the limits of the clear test material secreted by the zooid or its remnants. This inclusion in itself suggests a close association of the two, the bud and the epidermal tube with its contained epicardium.

An enlarged view of the same bud region is shown in Text-fig. 15A. All the tissues are clearly in a healthy state as indicated by the clear new tunicin secreted around the whole cylindrical system. The lower end of the epicardium extends to the fork where the two epidermal vessels divide, and is heavily congested with cells derived from its own wall, the epicardium being a completely closed sac or tube. Close to one side of the tip of the epicardium, within the tunicin sheath but external to the epidermis, lies the bud. It is a bud in its simplest shape and smallest dimension, and consists of an outer envelope of flattened epidermal cells similar to that of the adjacent zooid epidermis, and an inner more or less solid mass of cells identical in size and appearance with those of congested epicardial tip. A second example from the same region of the colony is shown in Text-fig. 15B, with a slightly smaller bud already isolated and the lower end of the epicardium turned up together with its enveloping epidermis. The upturning would be the inevitable result of internal extension of the epicardium beyond the fork of the epidermis. The upturned part survives, either as two or three buds successively constricted off, or by estrangulation at its base with constriction to form spheres occurring a little later. Several dozen cases similar to those illustrated were discovered and there seems to be little doubt that this is the normal manner of bud formation in *D. bermudensis*. The examples shown in Text-fig. 15C and D were found previously in a large colony of *D. magnilarva* but not fully understood at the time. Of those that are shown in Text-fig. 15, C is quite evidently the same event, namely, the turning up and subsequent constriction off of the lower end of the epicardium and adjacent epidermis. In Text-fig. 15D the constricted part appears lying in contact with the epidermal tube from which it in part arose, the non-turned part of the epicardium having disappeared,

and the turned-up part having undergone significant growth and differentiation.

There is little doubt that the process of turning up occupies a comparatively insignificant period of the whole growth cycle of the zooid, and the constriction off of this part an even shorter period. This brief event in the cycle, taken together with the fact that only a few regressing zooids can generally be found in any one colony at one time, undoubtedly accounts for its having been previously overlooked.



TEXT-FIG. 15. Bud formation from surviving epicardial tip of mature blastozooid.

A. Enlarged view of stage shown in Text-fig. 14E. B. Bud constricted from recurved tip of epicardium, with second bud forming. C, D. Probuds just isolated from parent epidermis. (A, B, *D. bermudensis*; C, D, *D. magnilarva*.)

The observations, however, are given additional plausibility in so far as the process just described is essentially the same as that described by many authors for bud formation in the tadpole larva. The difference is mainly in the time at which the process occurs, in the larva when the oozoid organization is elaborating and not yet functional, in the blastozooid only at the termination of the entire growth cycle. This difference may well be due to the difference in manner of development of the epicardium in the embryo and blastozooid, a possibility that will be discussed later, following a more detailed comparison between the two methods.

In Text-fig. 16 are shown six stages in the production of buds by the tadpole larva of *D. bermudensis*. For correlation with specific stages of the developing

tadpole as such, reference is made to Text-figs. 11 and 12. Bud formation occurs at the time the epicardia are growing as tubular structures evaginated from the floor of the pharynx. As the left epicardial sac grows ventrally to impinge upon the abdominal epidermis, the terminal epicardial epithelium thickens and proliferates, the adjacent epidermis doing the same, at least to the extent of conforming to the shape of the inner growing tissue.

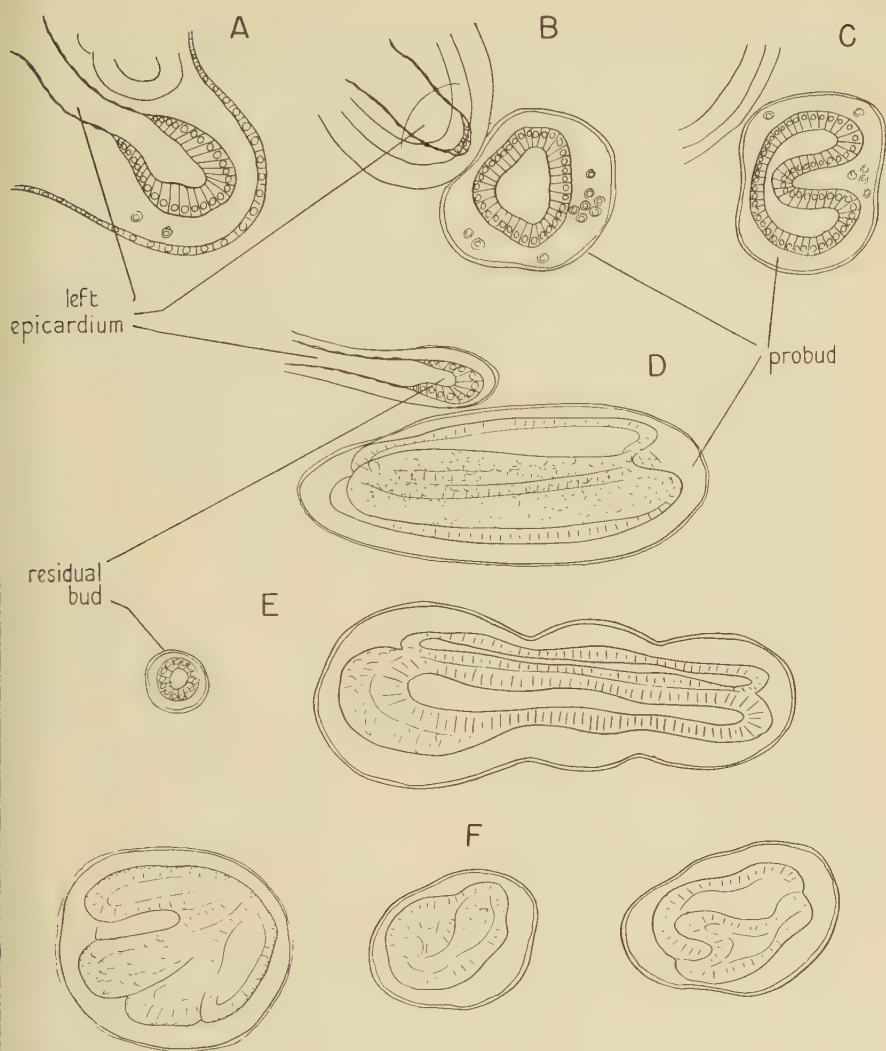
The first stage (Text-fig. 16A) consists of a hemispherical abdominal bulge involving the two tissue layers just described. An epidermal constriction separates off the greater part of the thickened epicardial tissue within the bulge to form an inner vesicle of cells derived from the epicardium and an outer one from the epidermis (Text-fig. 16B). Brien has shown that in *D. magnilarva* the constriction itself is due entirely to the action of the epidermis, the epicardium being purely passive in this respect. This has been confirmed for *D. bermudensis* as well, and also in *D. magnilarva* itself.

The double-walled sphere thus produced is, however, a pro-bud, that is, it is not as destined to give rise in its entirety to a single blastozoid, but to several true buds.

After separation of the pro-bud a number of form changes occur. The probud elongates along its original axis of growth as expressed by the epicardial axis until it becomes two or three times as long as it is wide. At the same time the inner vesicle invaginates along its longer axis, seen in optical end sections in Text-fig. 16C and 17B, and in lateral view in Text-fig. 16D. While this dual change takes place in the probud, the stump of epicardial and epidermal tissues of the parent zooid continues to elongate as a long tubular process, shown in Text-figs. 11B and 16D. At its tip the epidermis and especially the epicardial epithelium is thickened like the part originally constricted off as the probud. A little later this tip of growing tissue is in turn constricted off to form another double-walled sphere similar to the probud in its initial state except that it is relatively minute (Text-fig. 16E).

At the same time the probud attains its maximum length and constrictions appear in it initiated by the epidermis. These may appear simultaneously as two constrictions, as in Text-fig. 16E, or one may form as that shown in Text-fig. 17A, a second appearing somewhat later. In any case there is a definite tendency for the constriction nearest the original proximal end of the probud to appear first. The constrictions deepen until the entire elongated mass of the probud is divided into three more or less spherical buds. Of these the proximal bud has a mass markedly greater than the distal buds.

There are accordingly, as a rule, four buds produced in the tunic of the tadpole larva of *D. bermudensis*, the probud giving rise to one large and two medium-sized buds, the epicardial stump adding a fourth and comparatively minute bud. Of the four, only the largest shows a recognizable extent of morphogenesis at the time of the mature active tadpole. In this species the ultimate fate of the four has not been determined, but in the strictly comparable situation in *D. rosea* (p. 255) only the largest bud eventually develops



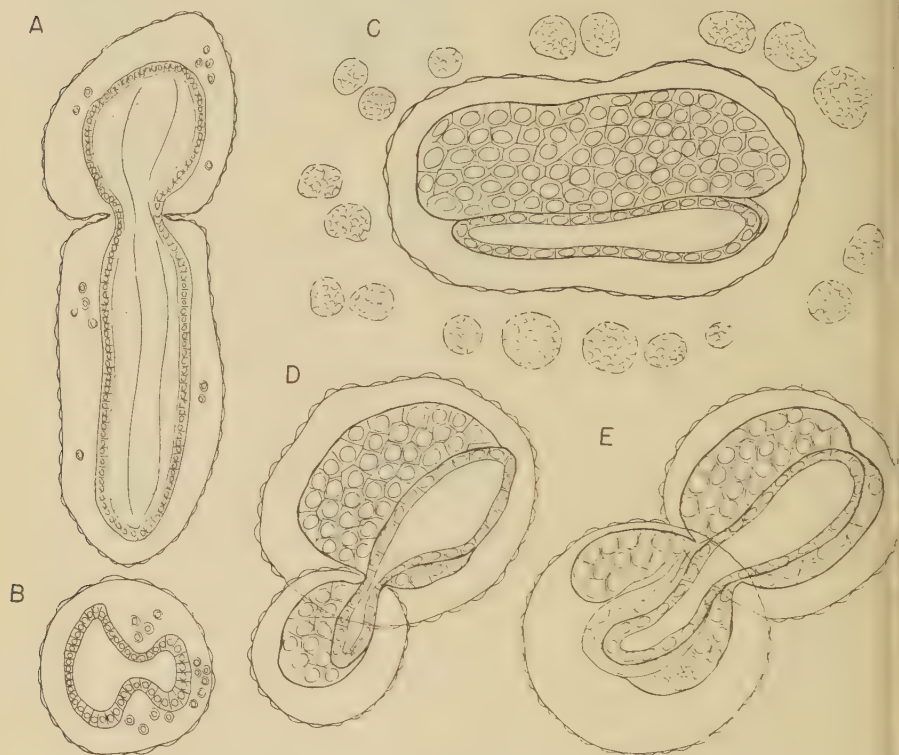
TEXT-FIG. 16. Bud production in tadpole of *D. bermudensis*. A. Initiation of probud as local outgrowth of tip of left epicardium and overlying epidermis. B. Segregation of probud. C. Doubling of epicardial component of probud. D. Elongation of probud and extension of residual epicardial tip. E. Strobilation of the probud and segregation of residual epicardial tip. F. Three definitive buds formed from probud.

to the functional state, the next largest attaining an early but arrested developmental stage, while the remainder disappear.

Probuds are to be seen both in the tadpole larva and in adult colonies. As such they have been reported in the larva and colony of *D. magnilarva* and *D. rosea*. They are equally typical of *D. garstangi* and *D. bermudensis* and the probuds of a mature colony of *D. garstangi* are shown. In each case it is clearly evident that the constriction of the probud into definitive buds is

entirely an epidermal action, the epidermis developing a progressive deepening constriction that strangulates the epicardial tissues within.

It is equally evident that the constriction appears in such a place that sphere is segregated, the size being determined by the short axis of the probud already formed. In the comparatively long probud of the larva at least three



TEXT-FIG. 17. Probuds. A. Probud of tadpole of *D. bermudensis* showing primary constriction. B. End view of same to show doubling of epicardial component. C. Probud of mature blastozooid of *D. garstangi* showing doubled and differentiated epicardial component. D, E. Similar probuds in process of unequal constriction.

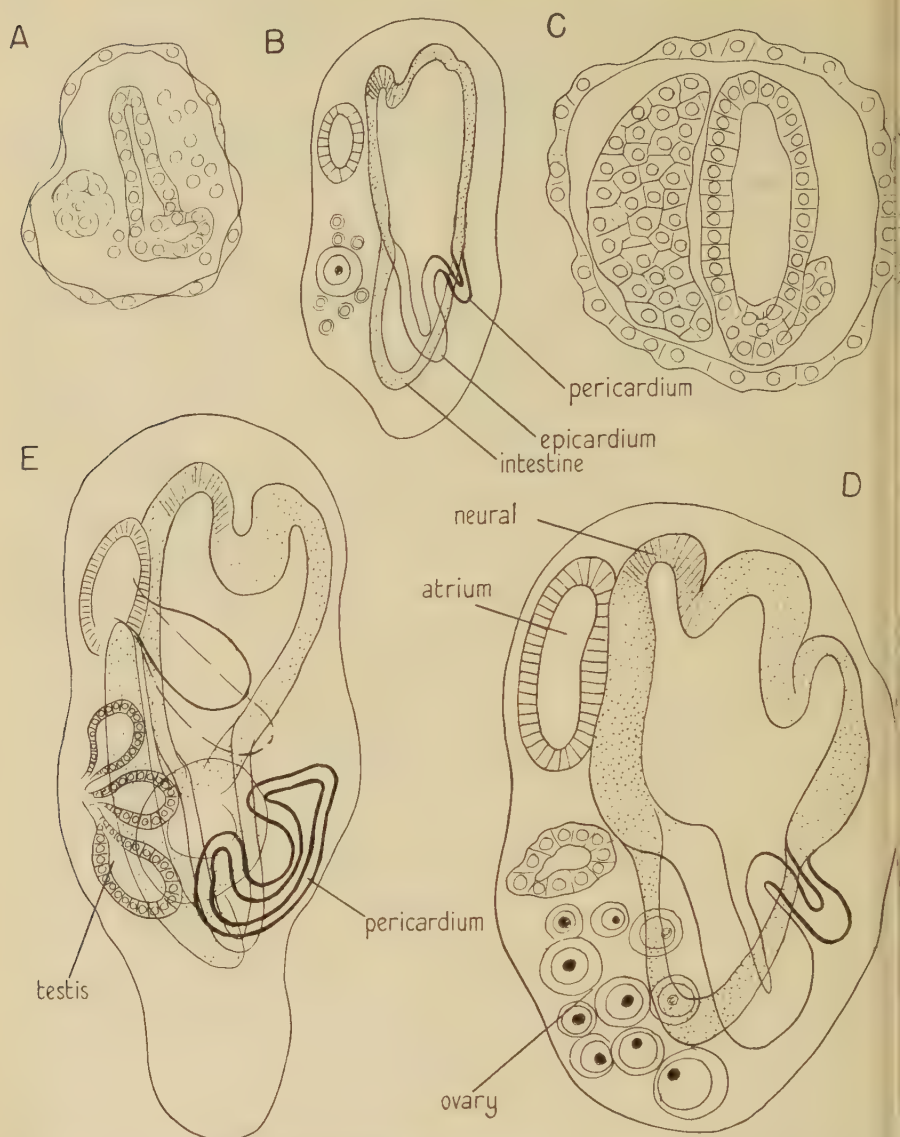
spheres are formed, but in the probuds of mature colonies the long axis rarely equals twice the diameter, the closest to this condition usually seen being that of Text-fig. 17C. The earliest stage in the constricting process is discernible as a slight saddle dividing the probud into approximately equal parts. When a probud is only slightly longer than it is wide, it is not divided equally but is constricted so that a maximum-sized sphere is cut off from one end, leaving a small residual sphere at the other (Text-fig. 17D). Frequently the epidermal layer of the probud is approximately twice as long as it is wide, but the contained epicardial component has a long axis that is considerably shorter than this. In such cases the epidermal constriction divides the outer layer to form epidermal vesicles of equal size, but the internal epicardial tissue

ying more towards one end than the other, is cut unequally (Text-fig. 17E). The epicardial component accordingly plays a passive role throughout with regard to the process of subdivision.

In Text-fig. 17C the probud is surrounded by numbers of large reserve-aden trophocytes. These tend to accumulate around all young buds and must be actively attracted to these positions. They are characteristic only of buds in established colonies, and have not yet been produced in the larva.

A further comparison may be made between the probuds found in larva and colony. In the larva the epicardial vesicle doubles or invaginates along its main axis as shown in Text-fig. 17B. Even in this case the larger division rapidly loses its lumen and becomes a solid tissue. In those of the colony the same doubling of the epicardial component occurs, but what was the smaller division is now the larger and consists of a compact mass of relatively large cells (Text-fig. 17C, D, E). The division of the epicardial tissue into these two distinct regions is definitely related to the morphogenesis of the definitive buds, and represents primarily a segregation of the reproductive and other mesodermal tissue from the rest. The significant feature here is that a basically important phase of morphogenesis has already commenced at a time when only two of the three dimensions of the organism are in existence. Essentially the same phenomenon occurs in the process of bud formation in *Salpa* and *Pyrosoma*.

It is evident therefore that *Distaplia* buds exhibit two variables, in the gross size of the bud and in the quantity of epicardial tissue. Those of the larva have a size range as a whole considerably smaller than those of the colonies, with only one bud large enough to ensure development. The others appear to be abortive as a result of being of too small an initial size, a phenomenon already recognized in the case of *Stentor* fragments (Lillie, 1896), *Hydra* (Papenfuss, 1934), *Clavelina* buds (Berrill and Cohen, 1936), and *Botryllus* (Berrill, 1941). Of the buds produced in colonies, both large and small divisions of a probud are usually capable of development, though large and small zooids are respectively formed. The relationship between size of equivalent stages and initial bud size is perhaps shown with greater certainty in comparing the development of the larval bud and the average large bud produced in a colony. Such a comparison is shown in Text-fig. 18, A and B being two stages in the development of the larval bud, and C and D exactly equivalent morphological stages of the colony bud. In Text-fig. 18A and C the bud is at its earliest stage of differentiation, very shortly following its segregation from the probud, and in each case consisting of an epidermal vesicle containing a hollow and a compact tissue mass. The hollow structure and the adjacent mass are both derived from the original epicardial sac. In Text-fig. 18B and D is shown an identical morphological stage representing in each case an equivalent degree of gross expansion or growth of the primary stage, yielding a large and small young bud respectively. In both small and large, however, the inner hollow structure has differentiated to form a neural



TEXT-FIG. 18. Early morphogenesis of bud of *D. rosea*. A, B. Two stages in development of bud of tadpole. C, D. Equivalent stages in development of relatively larger bud of mature blastozoid. E. Later stage in development of tadpole bud (i.e. first blastozoid generation). N.B. variability in gonad initiation.

evagination dorsally, and ventrally to form three evaginations representing the intestine, the epicardium, and the pericardium. In other words, the size of the primary stage when first constricted from the probud determines the size of subsequent stages up to the time the bud completes its development as such and becomes functional.

A further variable is in the relative mass of the central hollow vesicle and the adjacent compact tissue from which the gonads and some other tissues are derived, a contrast shown in Text-fig. 18A and c. The variable quantity of the presumptive gonad tissue seems to be the cause of the great variability in the nature of the gonads subsequently developed. The buds produced by the



TEXT-FIG. 19. A. Scheme of perpetual bud production by probud proposed by Brien. B. Alternative interpretation (for explanation see text). c. Strobilation of the probuds of *Colella pedunculata*, *C. racovitzai*, and *C. cerebriformis*, after Van Beneden and De Selys-Longchamps. (Probuds thin-lined, definitive buds thick-lined in A and B.)

zooid do not exhibit any discernible sign of gonads of either sex when fully developed, in the case of *D. rosea*, but early stages show rudimentary gonads of either sex though never both together (Text-fig. 18B and E). It has been shown elsewhere (Berrill, 1941) in the case of *Botryllus* that the initial size of the presumptive gonad area determines whether the bud subsequently developed is asexual, immature or mature male, or a mature functional hermaphrodite. The same relationship appears to hold here, and mature varieties develop only when the presumptive gonad initially segregated is quantitatively adequate. Submaximal masses may well give rise to main components only, as in *D. magnilarva*.

DISCUSSION

A very full review of the various interpretations of larval budding in *Distaplia* has been given by Brien (1939). It does not seem necessary to repeat this, other than to present his conclusions from this section (p. 113). These are that the very young larva emits a single bud, 'probud' or 'primordial bud' (Della Valle) formed by two concentric vesicles, an external vesicle derived from the medioventral ectoderm of the larva, an internal endoblastic vesicle, derived from the extremity of the left epicardium of the oozoid and between the two vesicles, mesenchymatous elements (Della Valle, Salensky, Julin, Caullery), which continue to divide in the colony (Caullery, Salfi), giving rise progressively to all the ascidiozooids of the colony.

Brien's own investigations are based upon this premiss, that the adult blastozooids have no power to bud and consequently the larval probud must give rise to all buds. With this in mind he interprets the succession of divisions of the probud in a most ingenious way, so that a series of both definitive buds and new probuds are continuously produced; in place of simple growth a single and unique proliferative stolon, successively liberating distal blastozooids, suffers a second constriction more proximal and almost synchronous with the distal blastogenetic constriction. This second proximal constriction should be called the proximal constriction of subdivision because it divides the proliferative stolon into two secondary proliferative stolons which will follow the same destiny as the primary proliferative stolon (Text-fig. 19A).

This concept does bring the budding process of *Distaplia* into line with that of *Colella*, and it does not conflict with the general picture of the probud and the definitive buds as seen in the tadpole larva, and is plausible if the fate of the probud subdivisions is unknown and if no other source of probuds or buds is known.

In the present account, in this connexion, the evidence is that of the several units produced by constrictions of the larval probud, the largest one or two develop into blastozooids and the smaller degenerate and disappear, there being no sign of probuds at the time of dissolution of the oozoid. Further, and of even greater significance, is the discovery that the adult blastozooids give rise to probuds, capable of producing at least two definitive buds, during the last phase of their own dissolution. There is accordingly no need to read a self-perpetuation process into the constrictions of the larval probud.

The activity of the probud is consequently interpreted here in a different and simpler manner. The definitive bud at its inception from the probud is from the hinder end that actually broke contact with the parent tissues. In the sense of origin this should be the proximal end, and the distal end the emergent epidermal-epicardial tip originally protruding from the parental abdomen.

The present account regards the probud as the constricted distal end of the abdominal outgrowth, separated off and continuing to elongate to a considerable extent after separation, and that the elongated probud undergoes further

constrictions to form spheres of sizes varying according to the probud diameter at the time. Only buds exceeding an initial critical size undergo further development, the rest atrophying. The large proximal (in our sense) bud develops, the most distal undergoes some morphogenesis but usually is abortive, while the smaller intervening buds never start (see Text-fig. 19B).

If Brien's reversal of the morphological proximal and distal ends of the probud were valid, the process of budding would be analogous and possibly homologous with that of the Thaliaceans mentioned above. In these forms constrictions occur in series from the distal end of the stolon and the proximal end remains attached to the parent. Actually in *Distaplia* constrictions commence at the opposite end, and the matching is therefore inaccurate. The nature of the stolon or probud from the point of view of origin, however, is significantly similar both with regard to tissues and site.

The relationship between *Distaplia* and *Colella* is another matter. These are genera clearly belonging to the same family. The budding process and morphogenesis of the bud are essentially the same in both. In the colonies of each, probuds in process of constriction are commonly seen, and in *Colella* as in *Distaplia* with no discernible connexion with a parent zooid. Consequently the question which is the original distal and proximal end of the relatively long probuds of *Colella* is debatable, but it is more plausible to consider them the same as in *Distaplia*. The origin of the probuds of *Colella* has been as obscure as that of *Distaplia* colonies and the explanation in the case of *Distaplia* may well be the explanation of both. This is supported to some extent by Herdman's figures of *Colella pedunculata* (1886, Pl. IX, figs. 3, 4, 5, 18), which are remarkably similar to the process illustrated here in Text-fig. 15.

The various species of *Colella* exhibit the same peculiar sexual conditions as *Distaplia* species, though shown in more extreme form. It is unfortunate that this exclusively subantarctic genus is so difficult to obtain, for it merits much more extensive investigation than has yet been possible. *Colella gaimardi*, *C. pulchra*, *C. concreta*, *C. elongata*, and *C. Thomsoni* have hermaphrodite gonads like those of *Distaplia bermudensis*, &c., but *Colella murrayi*, *C. ramulosa*, *C. quoyi*, *C. cerebriformis*, and *C. incerta* are said to be rigorously unisexual. The genus *Holozoa* (*Julinia*, Calman, 1895) is of equal interest and obscurity (cf. Hartmeyer, 1911), from the points of view of budding, sexuality, and life-cycle.

SUMMARY

The complete developmental cycle, sexual and asexual, is described for several species of the ascidian genus, *Distaplia*.

Part I. The cycle of *D. rosea* is described, from the active larva to the establishment of the first blastozooid generation. Of the four or five buds produced from the larval probud, only one develops, the rest remain abortive. Some larvae are often retained in the colony after the dissolution of the parent zooid and give rise to blastozooids (Text-figs. 1-3).

The general morphology of mature zooids is described for *D. bermudensis*, and their arrangement into typical systems in the colony. Zooids are at first functional males, while the brood-pouch develops later as the first egg in the ovary approaches maturity. The brood-pouch is essentially a loop of the oviduct and does not involve the atrial wall (Text-figs. 4-5).

In the gross development of the bud up to the time it becomes functional the originally spherical bud elongates relatively along its antero-posterior axis but otherwise does not exhibit differential growth. After the functional stage is reached, differential growth of the atrial siphon commences and eventually transforms the small circular siphon into the relatively enormous atrial aperture of the mature zooid (Text-figs. 4 and 6).

Gonads develop as hermaphrodite structures in buds of *D. rosea*, *D. bermudensis*, *D. occidentalis*, and *D. clavata*, but as either ovaries or testes alone in *D. magnilarva* (Text-fig. 7).

Part 2. Tadpole larvae of *Distaplia* vary greatly in size according to the species, but not in organization, the cell number of purely larval structures such as adhesive organs and sensory organs being constant (Text-fig. 8).

Cleavage of the egg, in spite of large size and yolk content, is typical of ascidians in general, and shows bilateral patterning comparable with that of *Styela* and *Ciona* (Text-fig. 9).

There is great growth in the development from the egg to the tadpole stage, though the full number of cells of both the notochord and tail muscle is reached at an early stage, about 40 in the case of the notochord (as in all other ascidians) and over 2,000 tail-muscle cells (the largest number known). In the later development of the tadpole larva, the anterior region bearing the adhesive organ and the ampullae becomes progressively constricted off until it remains attached only by a vascular stolon. The probud is separated from the abdomen at a precise stage in the whole development and subdivides before tadpole development is complete (Text-figs. 10-12).

The sensory vesicle contains a typical unicellular otolith and an ocellus consisting of optic pigment cup, layer of retinal cells, and three unicellular lens cells. In the tail the intracellular notochordal vacuoles run together to form a continuous fluid cylinder with the chordal cells forming a limiting sheath. Myofibrillae are continuous from cell to cell in the lateral interfaces of the ectoplasmic regions (Text-fig. 13).

Part 3. Contrary to all previous reports, buds are produced by adult zooids and are not confined to the tadpole larva, but they are produced at a different phase of the life-cycle of the individual zooid. After sexual reproduction is complete, the zooids atrophy, their tissues autolysing and becoming phagocytosed in part. All of the zooid degenerates with the exception of the epicardium and surrounding epidermis. This structure actually undergoes extensive elongation, at the close of which the posterior end of the pericardium and enclosing epidermis constricts off as one of two probuds (Text-figs. 14-15).

This is essentially the same process, apart from the stage with which it is associated, as occurs in the tadpole larva. In both cases the probud elongates

and constricts into two or more definitive buds (Text-figs. 16–17), the smallest of which fail to develop.

The formation of hermaphrodite or unisexual gonads depends primarily on the mass of the presumptive gonad tissue segregated at a very early critical period in bud morphogenesis (Text-fig. 18).

The process of bud formation is compared with that of the allied genus *Colella*, and an interpretation very different from that of Brien is presented (Text-fig. 20).

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An Egg-waxing Organ in Ticks

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With one Plate

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INTRODUCTION

MOST general accounts of the natural history of ticks include a brief description of the remarkable oviposition movements encountered in this group. As is well known, each egg, on leaving the vagina of the egg-laying female, is received for a few moments by a glandular organ—the organ of Génè—which is everted shortly before oviposition. The close connexion of this structure with oviposition is emphasized by the fact that it is absent

both from the male and from the immature stages. Its presence, however, is common to both families of ticks: in the Ixodidae it is everted from the space between the basis capituli and the scutum; in the Argasidae it appears from the camerostomal depression. No other groups of Acarina are known to possess the organ.

Nevertheless, despite the fact that this interesting structure was first described just one century ago, our knowledge of its morphology, and more particularly of its function, remains meagre. In one of the earliest accounts of oviposition in ticks, Gén   (1848) had noted that when the organ was pricked with a needle the eggs deposited near the vagina soon shrivelled. But he believed that the organ itself served as a receptaculum seminis. In a later experiment by Bertkau (1881), eggs were prevented from coming into contact with Gén  's organ by touching the latter with a glass rod and so causing it to retract at the critical moment. Such eggs, he found, subsequently shrivelled much more rapidly than eggs laid in the normal manner. These descriptions both refer to unidentified ticks, possibly *Ixodes ricinus*.

More recent work has been entirely descriptive. Lounsbury (1900) described how in *Amblyomma hebraeum* the organ 'gradually unfolds its glistening arms . . . grasps the egg and apparently envelops it in slime', and Wheler (1906) in a brief account of oviposition in *I. ricinus* also referred to the 'glutinous surface' of the organ. Nuttall (1908) mentioned that in *Haemophysalis punctata* the two vesicles of Gén  's organ contain a hyaline secretion but did not suggest a function. The internal glandular part of the organ has been variously referred to by other authors as the 'ovipositing gland', the 'cephalic gland' (Christophers, 1906), or as the 'subscutal gland' (Samson, 1909).

These descriptions suggested to us that the function of Gén  's organ is to provide the eggs with a waterproof covering—a function proposed indeed by Bertkau (loc. cit.) but never confirmed. Our studies, which are described in this paper, have shown that a waterproofing agent is undoubtedly transferred from Gén  's organ to the egg and that the agent in question is a wax. Now, although it is known that the integuments of many insects (Wigglesworth, 1945; Beament, 1945) and ticks (Lees, 1947) owe their waterproof properties to a thin superficial layer of wax which is secreted through the pore canals, the mode of transport of the wax through the cuticle remains obscure. The occurrence of a waterproofing organ, from which small quantities of material can be isolated, therefore provides a very favourable opportunity for examining the wax precursors. At the same time, however, a more detailed investigation of the structure of Gén  's organ has been needed in order to determine the precise site of secretion of the waterproofing agent.

Other questions have also been raised. The unusual nature of the waterproofing process called for a more extended examination of the properties of the wax in relation to those of the substrate, namely, the egg-shell. And this in turn has led us to follow the development of the egg-membranes and to examine some of their chemical and other properties.

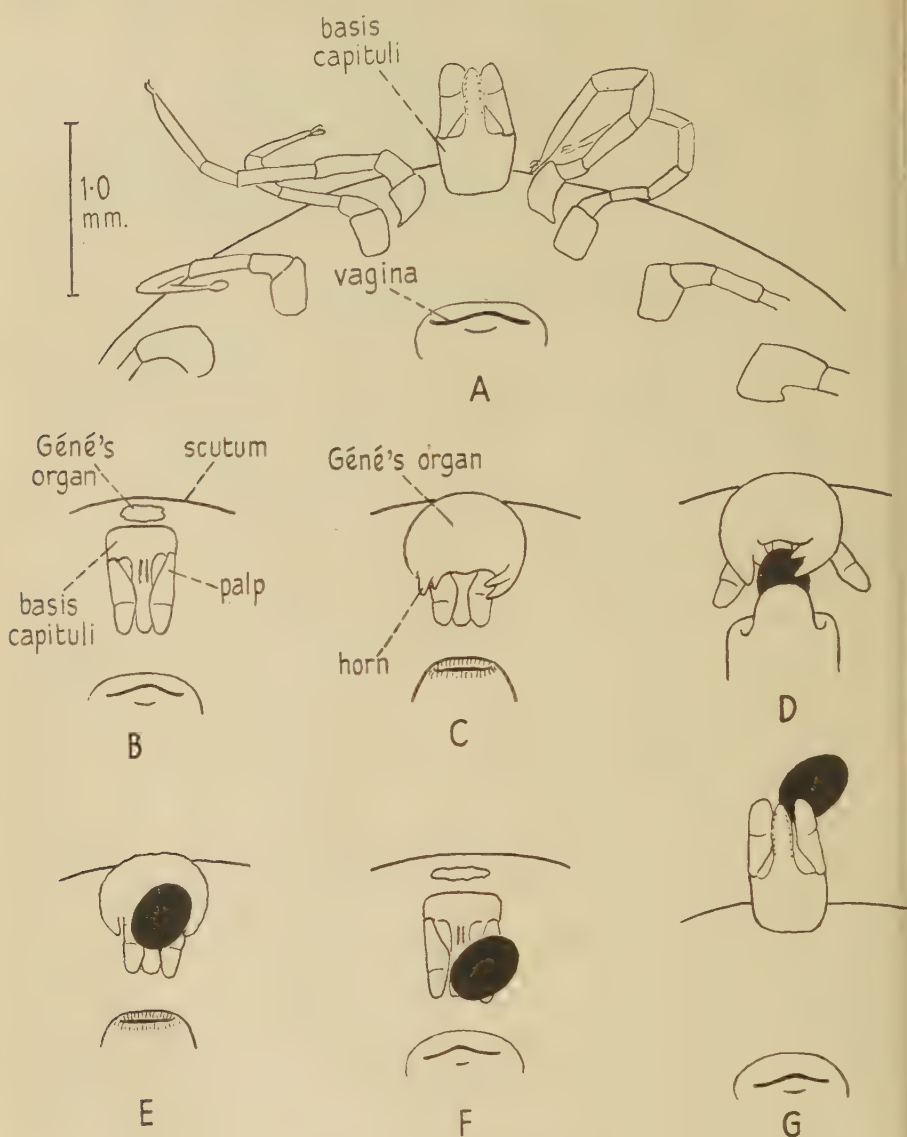
Most of our observations have been made on two species of ticks, *Ixodes ricinus* L. and *Ornithodoros moubata* Murray, which were selected as representative of the Ixodidae and Argasidae respectively. All ticks lay their eggs in large clusters. An engorged female *Ixodes* deposits a single cluster of some 1,000 eggs before she is spent. *O. moubata* lays, between successive blood meals, as many as 6 egg batches, each containing about 100 eggs. The small egg of *Ixodes* is elliptical in shape, the much larger egg of *Ornithodoros* ovoid or sub-spherical.

OVIPOSITION MOVEMENTS

Oviposition has been observed most closely in *I. ricinus*. Engorged female ticks were laid, ventral side uppermost, on the floor of a moist chamber and were secured in this position by means of plasticine bands; they were then left in darkness under a binocular microscope until egg-laying began. Mechanical disturbance or the access of light causes the suspension of oviposition, but usually a few eggs are laid in the light before the rhythm is interrupted. The sequence of events is illustrated in Text-fig. 1.

In the normal position of rest between the delivery of successive eggs, Gén 's organ is not visible, the capitulum is directed anteriorly, and the vagina is slit-like (Text-fig. 1A). The delivery of an egg is heralded by the downward movement of the capitulum, the tip of which describes a vertical arc and finally comes to lie closely applied to the ventral body-wall just in front of the vagina (Text-fig. 1B). Gén 's organ is then everted from between the dorsal posterior borders of the capitulum and the anterior margin of the scutum. Owing to the movement of the capitulum it protrudes ventrally and comes to lie over the hypostome and palps. The organ itself, when fully extended, is seen to consist of a balloon-like base which is produced on each side into two short horns which contain a translucent fluid (Text-fig. 1C). The whole organ is invested with an exceedingly delicate glistening cuticle, capable of a high degree of folding.

When oviposition is about to take place the hypostome is tucked firmly against the ventral body-wall, the palps are splayed out, and Gén 's organ is inflated to its maximal extent. At the same time the inner lining of the vagina begins to unroll like the finger of a glove (Text-fig. 1D). The prolapsed vagina or 'ovipositor' forms a tube which, when fully extended, nearly touches Gén 's organ and is a most effective instrument for delivering the egg. After depositing the egg between the horns, the 'ovipositor' is quickly retracted. Gén 's organ with the egg attached is then partially deflated and inflated several times and the palps are often worked sideways in an active manner. Finally, the organ is retracted completely within the body and the egg is left adhering to the dorsal surface of the hypostome (Text-fig. 1E, F). The cycle is completed when the hypostome swings into the dorsal position again, carrying the egg with it (Text-fig. 1G). Because of these events the eggs tend to accumulate on the dorsum of the tick and not on the ventral surface, and as

TEXT-FIG. 1. Oviposition movements in *Ixodes ricinus*.

the egg-laying female rarely moves after the onset of oviposition the eggs are deposited in a dense cluster.

The rate of egg production under favourable conditions is not easy to determine, as after any disturbance laying is not resumed for some hours. One female tick laid as many as 246 eggs during 48 hours, that is, at the rate of 1 egg every 12 minutes. According to Wheler (1906), however, the sheep tick may sometimes lay eggs at the rate of 1 every 3 minutes.

The movement of the palps and of Gén  's organ itself may sometimes have the effect of rotating the egg and so promoting increased contact with the shell. However, careful observation has convinced us that as a general rule only a small area of the shell—certainly less than half the total surface—establishes contact with Gén  's organ. At this time the egg usually lies between the horns which may embrace it as the organ is retracted. Nevertheless contact with the horns is not essential, for eggs which chance to be deposited on the dorsal aspect of the organ, where the horns cannot reach them, undergo normal development subsequently. Once oviposition is under way the cuticle of Gén  's organ soon acquires a greasy glistening appearance and the film of grease gradually extends to the legs and the cuticle near the vagina. However, spreading of the grease to the egg-shell cannot be observed under the microscope.

The oviposition movements in *Ornithodoros moubata* have not been followed in detail as this tick is even more sensitive to disturbance, but the appearance of females which have been interrupted at different stages of egg-laying suggests that the process is the same in all essentials. Co-ordinated movements involving the eversion of Gén  's organ, the prolapse of the vaginal lining, and the rotation of the hypostome probably take place as in *Ixodes*. Since the hypostome is shorter, however, and the eggs less sticky, the latter are not carried dorsalwards but instead accumulate in a heap beneath the anterior region.

VISUAL DEMONSTRATION OF THE SECRETION OF WAX BY G  N  'S ORGAN

The role of Gén  's organ in secreting a wax is most simply demonstrated in *O. moubata*. In this species the organ can be caused to evert if the body of the tick is compressed between two microscope slides so that only the proboscis and camerostome project freely (Pl. I, fig. 1). After the slides have been clipped together with Cornets forceps the organ can be held everted for further detailed examination. A full description will be deferred until a later section.

In the normal egg-laying female the surface of Gén  's organ is always devoid of large accumulations of wax. If, however, engorged ticks are kept for 3 or 4 weeks at 15   C.—a temperature just too low for oviposition—large amorphous deposits of wax appear on the surface of the cuticle, usually around the base of the horns. These can easily be collected on a needle after everting the organ by pressure. Wax accretions on three organs are shown in Text-fig. 2; not all individuals secrete wax in such quantity.

That Gén  's organ in *Ixodes* is also concerned in secreting a wax can be shown by everting the organ and touching it against a clean glass slide. The demonstration is less striking than in *Ornithodoros*, however, for in this species the wax fails to accumulate in any quantity owing to its greater mobility (see p. 302).

THE FATE OF EGGS LAID OUT OF CONTACT WITH GÉNÉ'S ORGAN

The function of Géné's organ in waterproofing the eggs has been confirmed by occluding the opening through which the organ is everted and following the subsequent fate of the eggs when exposed to different humidities. In *Ornithodoros*, eversion was prevented by covering the camerostomal fold and proboscis with cellulose paint. This somewhat delays the onset of oviposition but afterwards egg-laying continues at the normal rate. The procedure adopted was to allow the same ticks, isolated singly in specimen tubes, to lay successive small batches of eggs with the opening free, then occluded, then



TEXT-FIG. 2. Natural accumulations of wax on Géné's organ in *Ornithodoros*.

finally free again. The laying ticks were exposed to a humidity of 70 per cent. R.H. in an incubator at 25° C. where the eggs remained undisturbed for 14 days. At this temperature all normal eggs hatch within 12 days.

Some of the results obtained are given in Table 1. As a general rule (e.g. ticks, nos. 1-3) very nearly all the eggs laid with Géné's organ free hatch normally, while those laid without the intervention of the organ are all completely shrivelled and hard. Sometimes, however, a tick which at first lays normal eggs continues to lay shrivelling eggs after Géné's organ has been freed (e.g. no. 4). This may be ascribed to the disturbing influence of covering and uncovering the camerostome which probably upsets the synchronous eversion of the organ. The eggs failing to touch Géné's organ will therefore remain unwaterproofed. A few shrivelling eggs are sometimes laid among or preceding an otherwise normal batch (e.g. no. 1). No doubt this is also caused by an occasional faulty eversion of Géné's organ. The same phenomenon is seen when egg-laying ticks are kept in crowded cultures where mutual disturbance is often sufficient to lead to the production of large numbers of unwaterproofed eggs.

Unless contact with Géné's organ influences the viability of the egg in other ways, a proportion of the eggs deprived of contact would be expected to hatch if the atmosphere were kept sufficiently moist. A further group of egg-laying females with Géné's organ obstructed was therefore set aside in saturated air and was examined only when hatching should have been complete. Six females laid a total of 386 eggs of which 23 hatched, the remainder, even in

the damp atmosphere, gradually collapsing and darkening. One hundred and eighty-four eggs, which were laid by 3 ticks, yielded no nymphs. One female, on the other hand, laid 28 eggs of which as many as 14 hatched. Normal egg-masses kept in a damp atmosphere remain free from moulds almost indefinitely. It was noteworthy that eggs laid without the intervention of Géné's organ rapidly acquired a thick felt of hyphae growing on the surface of the egg-shell. This is a further result of the absence of wax from the shell.

Parallel observations have been made on *Ixodes ricinus*, with generally similar results (Table 1). Egg-laying females were secured on their backs with

TABLE 1. *The hatching of successive egg-batches laid with or without the intervention of Géné's organ*

The eggs of *O. moubata* and of *I. ricinus* were exposed during incubation to humidities of 70 and 100 per cent. R.H. respectively

Species	Tick no.	Géné's organ uncovered			Géné's organ blocked			Géné's organ again uncovered		
		No. of eggs			No. of eggs			No. of eggs		
		Laid	Hatched	Shrivelled	Laid	Hatched	Shrivelled	Laid	Hatched	Shrivelled
<i>Ornithodoros moubata</i>	1	38	36	2	64	0	64	49	49	0
	2	22	22	0	47	0	47	53	46	7
	3	36	36	0	25	0	25	82	45	37
	4	22	22	0	70	0	70	47	0	47
<i>Ixodes ricinus</i>	5	998	984	14	308	20	288	579	566	13
	6	859	822	37	255	0	255	604	486	118
	7	218	208	10	247	2	245	555	213	342
	8	171	151	20	154	10	144	216	73	143

the hypostome firmly embedded in plasticine. As this eliminates the hypostomal movements, as well as preventing the eversion of Géné's organ, the eggs collect in a pile round the opening of the vagina. Normal egg batches of this species are far more susceptible to desiccation than are those of *Ornithodoros* (p. 301) and for complete hatching must be exposed continuously to saturated air. The results of observations on the hatching of small egg-clusters, laid consecutively, with or without the aid of Géné's organ, are summarized in Table 1. Again, as the first column shows, there is always a small number of shrivelling eggs in every normal egg-cluster, probably resulting from the failure of the organ to evert properly. With the organ blocked the great majority of the eggs, even in the saturated atmosphere, gradually collapse and darken and very few hatch. After removal of the block greater numbers of eggs hatch, although in many individuals an abnormally high proportion of the eggs prove to be non-viable. As in *Ornithodoros*, therefore, the interference with Géné's organ appears to hinder its proper functioning, even when the obstruction has been completely removed.

DEMONSTRATION OF THE WATERPROOFING LAYER ON THE EGG

If, as has been suggested, a waxy covering is applied to the outside of the egg by Géné's organ, wax solvents and detergents should be capable of

attacking this superficial layer and therefore of exerting a pronounced effect on transpiration through the shell. As Table 2 shows, this expectation is fulfilled.

TABLE 2. *The effect of chloroform and Co9993 on the water loss from small egg-batches (average weight 26 mg.) laid during the previous 24 hours*

Species	Treatment	Per cent. loss of weight during 1 hr. in dry air at 25° C.
<i>Ornithodoros moubata</i>	None	0·7
	Extracted in chloroform at 15° C. for 1 minute	45·0
	Smeared with Co9993	24·0
<i>Ixodes ricinus</i>	None	1·2
	Extracted in chloroform at 15° C. for 1 minute	51·0
	Smeared with Co9993	15·7

Water loss from newly laid egg-masses is greatly increased if they are extracted with cold chloroform or smeared with the wax emulsifier Co9993 (see Wigglesworth, 1945). The effect of chloroform is particularly dramatic on the relatively small eggs of *Ixodes*. Of the normal waterproofed eggs about 50 per cent. shrivel during 24 hours in dry air at 25° C. After washing in chloroform for 30 seconds all the eggs shrivel and dry out completely within 5 minutes. As a result of the removal of the wax, the eggs no longer stick together and tend to roll about freely.

That Géné's organ is concerned with the deposition of the wax layer can be shown by extracting, with chloroform, egg batches which have been laid with or without the intervention of the organ; the relative effect of this treatment on water loss can then be compared. Results with *Ornithodoros moubata* and *Ixodes ricinus* are set out in Table 3. The number of eggs that are shrivelled after a given interval of time can be used as a convenient index of water loss. As it has been found that the permeability of *Ornithodoros* eggs, when devoid of the wax layer, may differ conspicuously from one batch to another, comparisons have always been made using eggs deposited by the same tick.

Normal eggs of *Ornithodoros* never show the slightest shrivelling after 24 hours in dry air at 25° C., while those washed with chloroform are all shrivelled, and some are dry, after only 30 minutes (Table 3). Eggs laid in moist air without the intervention of Géné's organ also shrivel rapidly in dry air; and the rate of shrivelling is not increased after chloroform treatment. Extraction with chloroform also makes little difference to the rate of water loss from eggs dissected from the uterus. Thus it is clear that in this species, the waterproofing material on the outside of the egg is derived solely from Géné's organ.

This is not the case with *Ixodes*, however. Egg-masses deposited with

Géné's organ covered are certainly more permeable than those laid with the assistance of the organ. Thus after 4 hours in dry air at 25° C. none of the eggs in a normal batch of 50 were shrivelled and only a few had begun to imple, whereas of the eggs laid by the same tick without the organ about half were completely shrivelled (Table 3). Nevertheless, the permeability of the eggs laid without Géné's organ is greatly increased by washing in chloroform; shrivelling is then as rapid as it is after extraction of normal eggs. These results indicate that the egg, before establishing contact with Géné's organ, must already have acquired a covering of some chloroform-soluble material capable of reducing the permeability of the shell. Eggs dissected from the oviduct, on the other hand, are highly permeable; and the permeability is not noticeably increased by chloroform (Table 3).

TABLE 3. *The effect of extracting eggs from various sources with chloroform at 15° C. for 1 minute*

The rate of shrivelling after treatment was observed in dry air at 25° C.
All laid eggs were from batches less than 24 hours old

Species	How laid	Treatment	No. of eggs	No. of eggs shrivelled after			
				5 min.	30 min.	4 hrs.	24 hrs.
<i>Ornithodoros moubata</i>	With Géné's organ	None	20	0	0	0	0
		Chloroform	20	14	20	..	..
	Without Géné's organ	None	20	8	20	..	..
		Chloroform	20	9	20	..	..
	Eggs from uterus	None	20	16	20	..	..
		Chloroform	20	15	20	..	..
<i>Ixodes ricinus</i>	With Géné's organ	None	50	0	0	0	23
		Chloroform	50	50	..	..	..
	Without Géné's organ	None	50	0	5	24	50
		Chloroform	50	50	..	..	..
	Eggs from oviduct	None	50	50	..	..	..
		Chloroform	50	50	..	..	..

It follows that the egg of *Ixodes* is waterproofed in two stages: the egg receives first an external covering of waterproofing material during its passage down the common oviduct and vagina, and then acquires an additional coating from Géné's organ. Further evidence, which is presented below, suggests that both waterproofing agents are waxes with very similar, if not identical, properties. This two-stage application of lipoid can easily be detected if egg-masses laid by the same tick, with and without the intervention of Géné's organ, are compared under the binocular. It is then obvious that although both are visibly greasy, the former are provided with more liberal quantities of wax. The eggs from the oviduct have, in contrast, a smooth, highly polished appearance with no trace of grease.

The greater permeability of eggs that are prevented from touching Géné's organ is due principally to the fact that the first wax layer is always incomplete.

Since the egg-shell contains reducing substances (p. 323), this can be demonstrated by immersing the eggs in 5 per cent. ammoniacal silver nitrate. The shell and yolk of normal eggs always remain completely unstained by this treatment, whereas eggs that have failed to touch the organ invariably show some staining of the shell but little blackening of the yolk. After washing in chloroform there is widespread staining of the shell and very rapid blackening of the yolk as the stain penetrates into the interior of the egg. Whether the thickness of the wax layer also influences the permeability has not been determined.

The eggs laid by different females with Gén 's organ out of action vary greatly in permeability. The following is an example. The eggs deposited by 2 ticks on 3 consecutive days were collected and exposed separately to dry air at 25  C. for 4 hours. The egg batches of the first yielded 10, 5, and 2 per cent. of shrivelling eggs, those of the second 35, 44, and 49 per cent. respectively. Individual differences in the coverage of the eggs with wax are probably the cause of this variability.

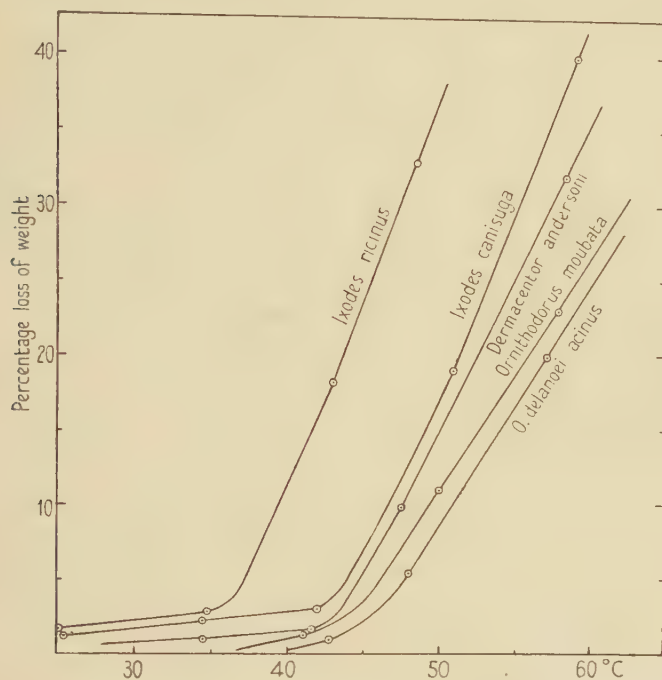
The presence of the first coating of wax, conferring on the egg a certain degree of impermeability, may account for statements in the literature to the effect that Gén 's organ is unnecessary for normal oviposition. Smith (1945) records an example of a specimen of *I. dentatus* which came away from the host with so large a piece of skin still attached to the hypostome that the eversion of Gén 's organ was prevented. Yet the eggs deposited by this female appeared normal. With *I. ricinus*, however, we have shown that the first wax covering is insufficient, even in damp air, to prevent the collapse of most of the eggs over the long period required for normal development.

THE EFFECT OF TEMPERATURE ON WATER LOSS FROM THE EGG

Previous work with insects (Ramsay, 1935; Wigglesworth, 1945; Beament, 1945), with ticks (Lees, 1947), and with an insect egg (Beament, 1946b) has shown that the waxes which are responsible for the impermeability of the cuticle or egg-shell undergo transitional changes at a certain 'critical temperature' and permit water to pass more readily. An evaporation curve exhibiting a pronounced break at a certain temperature can therefore provide confirmatory evidence that a wax layer is present. But in addition it has been found that the critical temperature bears a close relation to the other physical properties of the waxes, which in fact show great variability in different species. Among ticks, species from dry environments, such as the Argasidae, have low rates of transpiration and high critical temperatures; whereas the reverse is true of species from damper environments, like the majority of the Ixodidae.

The effect of temperature on water loss has now been investigated in a representative series of tick eggs. Owing to the small size of the egg it is impracticable to estimate water loss in relation to surface area; for the present purpose of determining the approximate critical temperature it has sufficed to plot loss of weight against temperature. The usual experimental procedure adopted was to expose small clusters of eggs taken from a single egg-mass (weighing about 20 mg. and containing up to 200 eggs) to dry air at different

temperatures for periods of 30 minutes. The eggs were contained in a small gauze basket which was suspended over phosphorus pentoxide in a conical flask immersed up to the neck in a water bath. Owing to the rapid desiccation above the critical temperature, fresh lots of eggs from the main egg-cluster were often used to obtain separate points on the curve.



TEXT-FIG. 3. The effect of temperature on the evaporation of water from the eggs of different species of ticks.

The evaporation curves for the eggs of five species are shown in Text-fig. 3. The rate of water loss from egg-masses of comparable weight after exposure to dry air at 25° C. for 24 hours and the approximate critical temperature of the eggs are recorded in Table 4. We also include for comparison the critical temperatures previously obtained for the cuticle of the parent species (Lees, 1947). It should be borne in mind, however, that the values for the cuticle were read off from curves relating transpiration and surface area.

The results bring out several points. First, the different species can be arranged in a graded series according to the resistance of the eggs to desiccation at 25° C. When this is done, the sequence corresponds closely with a similar series based on the order of resistance of the adult tick itself (Lees, 1947). In other words, susceptible species, such as *Ixodes ricinus*, also lay susceptible eggs; more resistant species, like *Ornithodoros*, lay comparatively resistant eggs, and so on. Secondly, the critical temperature is related to the rate of water loss below the critical temperature (e.g. at 25° C.). Thirdly, the

critical temperature of the egg in Ixodidae is nearly identical with that of the cuticle (the difference of 3°C . in the case of *I. ricinus* is of doubtful significance); whereas the critical temperature of the egg in Argasidae is much lower than that of the cuticle. In *O. moubata*, for example, we obtained values of 45°C . and 62°C . for the egg and cuticle respectively. The egg critical temperature is only 1°C . above that of the most resistant Ixodid, *Hyalomma savignyi*.

TABLE 4. *Water loss from small batches of eggs (weighing about 20 mg.) and their approximate critical temperatures*

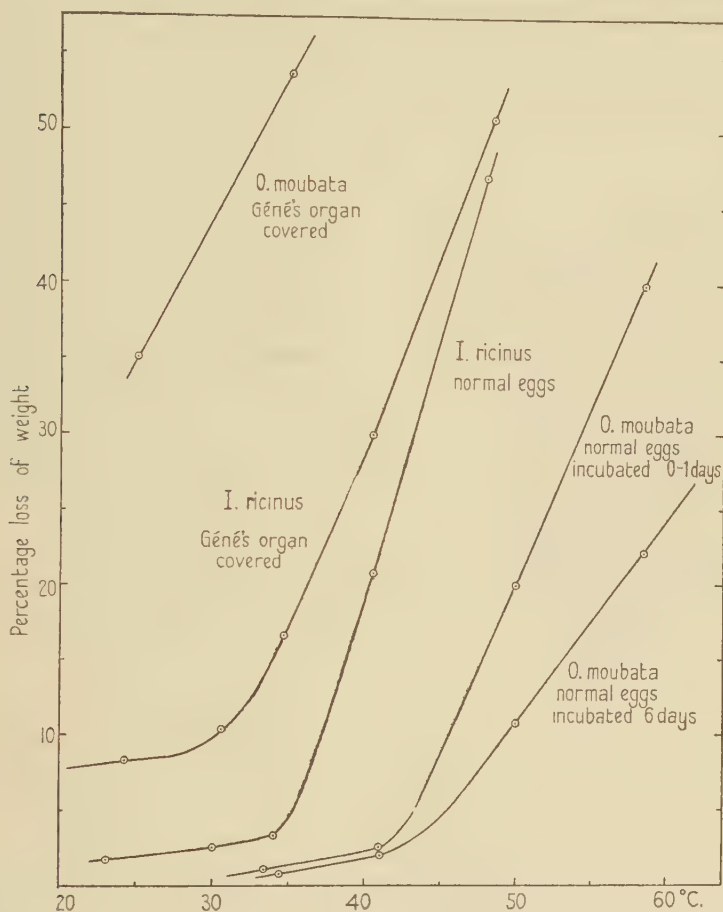
The critical temperature of the female ticks are included for comparison

Family	Species	Per cent. loss of weight in dry air during 24 hrs. at 25°C .	Critical temperature, $^{\circ}\text{C}$.	
			Egg	Cuticle
Ixodidae	<i>Ixodes ricinus</i>	27.2	35	32
	<i>I. canisuga</i>	15.6	42.5	42
	<i>Dermacentor andersoni</i>	9.0	43	44
	<i>Hyalomma savignyi</i>	8.0	44	45
Argasidae	<i>Ornithodoros moubata</i>	7.9	45	62
	<i>Ornithodoros delanoei</i>			
	<i>acinus</i>	2.5	46	67

Besides differing in their transitional temperatures, the egg waxes in this range of species probably exhibit parallel differences in their other physical properties. We have already recorded that in *I. ricinus* the lipid secreted by Gén 's organ is a labile grease, whereas in *O. moubata* it is a viscous wax. Species with intermediate critical temperatures no doubt possess egg waxes with intermediate physical properties. A difference in 'stickiness' can indeed be easily appreciated merely by handling the egg-masses, for the eggs adhere to one another solely by virtue of their waxy covering. The egg-masses of *I. ricinus*, for example, stick together most tenaciously; those of *H. savignyi* appear much less greasy and fall apart quite readily; while the eggs of *O. moubata* and *O. delanoei* show only the slightest mutual coherence.

We have already pointed out that in the *Ornithodoros* egg the entire waterproof covering is supplied by Gén 's organ, whereas the *Ixodes* egg is partly waterproofed before leaving the vagina. Confirmation is provided by the effect of temperature on the water loss from egg-masses of the two species laid with and without the intervention of Gén 's organ. Eggs of *Ornithodoros* laid in a damp atmosphere with the organ covered lose water rapidly at all temperatures when exposed to dry air (Text-fig. 4). *Ixodes* egg-masses, laid without Gén 's organ, although distinctly more permeable than eggs touched by the organ, still show a definite break in the evaporation curve at about 31°C . (This type of curve may be compared with those obtained by Wigglesworth (1945) for soil-inhabiting larvae of *Tipula* and *Hepialus*. Although the cuticular wax layer of these insects is more or less severely scratched by soil particles, a distinct break in the evaporation curves may still be detected.

Normal eggs laid by the same ticks had a critical temperature of about 34°C . (Text-fig. 4). This is additional evidence that the partial waterproofing effect is also due to the presence of a wax layer. Probably the wax is similar in nature to that secreted by Gén  's organ, although smaller in quantity.



TEXT-FIG. 4. The effect of temperature on the evaporation of water from egg-masses laid with and without the intervention of Gén  's organ.

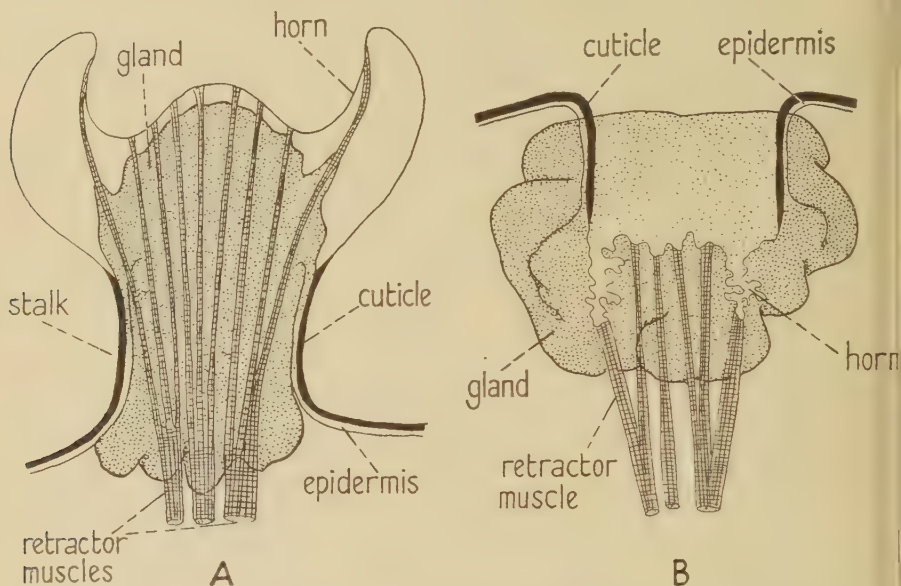
Text-fig. 4 also shows the evaporation curves of *Ornithodoros* eggs 0-1 days after laying and after incubation for 6 days at 25°C . Eggs of both ages have a critical temperature of about 44°C . But the older eggs lose water at a lower rate, particularly at temperatures above 44°C . Some possible explanations of this effect are discussed later (p. 324).

THE MORPHOLOGY OF G  N  'S ORGAN IN *ORNITHODORUS MOUBATA*

The external appearance of G  n  's organ in *Ixodes* has already been described briefly. Because of the greater ease of everting the organ in

Ornithodoros, we have chosen this species for more detailed study. Much of the internal structure is visible through the transparent cuticle.

The everted organ consists of a broadly sessile stalk surmounted by two large crescentic horns (not four as in *Ixodes*). When it is fully inflated the stalk is completely occupied by the white tissues of the gland (Text-fig. 5 A). The horns are particularly conspicuous as they are usually filled with a clear refractile liquid—evidently the secretion of the gland—which extends also round the margin of the organ at the base of the horns and occupies the space



TEXT-FIG. 5. Géné's organ in *Ornithodoros moubata* (schematic). Optical sections of the organ in the everted (A) and retracted (B) condition.

between the gland and cuticle. The amount of secretion in the horns varies considerably: sometimes there is only a little at the tips and the gland then extends well into the base of each horn.

On retraction the entire organ is pulled through the narrow slit in the camerostome and turned inside out. The gland then comes to lie in a series of pleats inside the body-cavity, while the cuticle of the stalk and horns is contained in a highly folded state within the gland itself (Text-fig. 5B).

Géné's organ has a powerful system of retractor muscles which penetrate between the cells of the gland and run back through the stalk to insertions in the dorsal body-wall. Most of the distal attachments of these muscles are found on the cuticle between the horns, but each horn is provided also with a single retractor attached near the tip (Text-fig. 5B). These observations resolve the difficulty experienced by Robinson and Davidson (1914) in accounting for retraction. These authors, who studied only the invaginated organ in *Argas*

persicus, believed that the retractor muscles were attached only to the epidermis or gland ('hypodermal sac') and not to the cuticle ('chitinous sac').

The mechanics of the process of eversion have not been made out with certainty. Gén $\acute{e}$'s organ in *Ornithodoros* is devoid of any intrinsic musculature capable of causing eversion. There is, therefore, a certain resemblance with the blow-fly ptilinum which also lacks intrinsic protractors (Laing, 1935). On the other hand, there are no externally visible signs such as pulsations of the cuticle which would suggest that Gén $\acute{e}$'s organ, like the ptilinum, is inflated by locally increased blood-pressure. Robinson and Davidson held that eversion is brought about by the rapid secretion of fluid between the 'hypodermal' and 'chitinous' sacs. But this again is unlikely as the amount of secretion in the horns during normal oviposition appears to be no greater at the moment of eversion than at any other time between the successive delivery of eggs when the organ is retracted. It is more probable that a number of indirect muscles are concerned: the most important is certainly the depressor of the hypostome which is also inserted on the dorsal body-wall just posterior to the point of insertion of the gland retractor. This muscle appears to form a partial septum which, as it shortens and depresses the hypostome, impinges against the invaginated gland. It is noticeable that the organ can be everted with a much smaller application of pressure if the hypostome is held simultaneously in the depressed position.

Nuttall (1908) mentions that the inflated vesicle of Gén $\acute{e}$'s organ in *Haemophysalis* is covered with minute punctuations which he took to be pits from which the secretion of the organ escaped. In *Ornithodoros* papilla-like processes are sometimes present near the base of the horns. Examination of serial sections showed, however, that these corresponded merely to the points of the muscle insertions. There is no specialized channel by which the secretion can escape through the cuticle of the organ. We consider this question in greater detail below.

THE SITE OF WAX SECRETION ON G $\acute{E}$ N $\acute{E}$ 'S ORGAN

Although, under certain conditions, large external deposits of wax may be formed (p. 295), the points of secretion cannot easily be determined by inspection owing to the spreading propensities of the wax. The internal structure has, therefore, been examined in greater detail with the object of determining the site of transfer through the cuticle.

Sagittal and horizontal sections of whole organs fixed in the everted state show clearly that the gland is actually a specialized region of the epidermis which is continuous with the epidermis underlying the general body cuticle (Pl. I, fig. 2). There is no differentiation into 'hypodermal sac' and 'gland' such as Robinson and Davidson (1914) describe. At the base of the stalk the epidermal cells are relatively attenuated but at a point just proximal to the base of the horns the epidermis becomes greatly thickened and is folded inwards, so forming the pleats and convolutions of the gland.

The relations of the gland and epidermis to the cuticle are as follows: Over the basal region of the stalk the epidermis is closely applied to the cuticle, but near the point where the unspecialized epidermis passes into the thickened glandular tissue it becomes detached from the cuticle, thereby leaving a conspicuous lumen wherein the secretion of the gland accumulates (Text-fig. 5A; Pl. I, fig. 2). We have already stated that neither gland nor epidermis as a rule extends far into the horns. The walls of the horns were examined carefully in sections stained heavily with iron haematoxylin, but no trace of an inner cytoplasmic lining could be detected. There seems no doubt, therefore, that the secretion of the gland must be regarded as extracellularly accumulating between the cell wall and a part of the cuticle which is non-living.

The histology of the gland epidermis is shown in Pl. I, figs. 3 and 4. The component cells are columnar or wedge-shaped with well-defined cell boundaries. The nuclei lie near the margin of the cell abutting the haemocoel. In egg-laying females the gland cytoplasm is often distended with droplets, some of which appear to be on the point of discharge into the lumen (Pl. I, fig. 3).

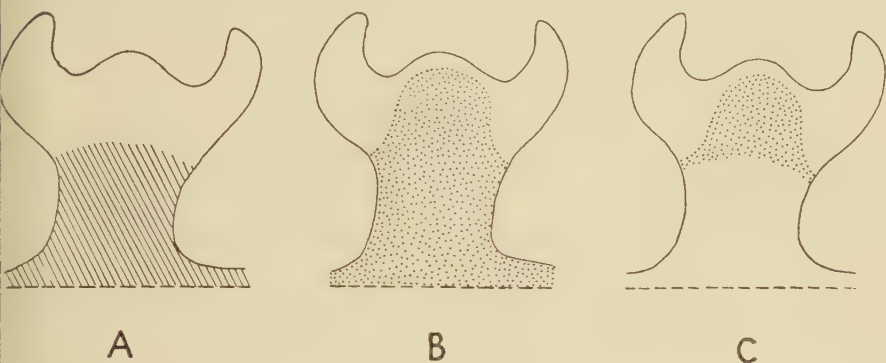
The cuticle investing Gén 's organ includes layers of smooth epicuticle and of endocuticle which pass without interruption into the cuticular layers of the general body-wall. The endocuticle is about $20\ \mu$ in thickness over the stalk region, but at those points where the secretion is stored it becomes very attenuated, only attaining a thickness of about $5\ \mu$ in the horns. Sections, cut at $6\ \mu$ and mounted in tap-water, were examined with an oil immersion objective for the presence of pore canals. Their approximate distribution is shown in Text-fig. 6B. Although there are numerous pore canals traversing the endocuticle of the stalk, they are more sparsely distributed near the point at the base of the horns where the epidermis becomes detached from the cuticle and pore canals are entirely absent from the endocuticle of the horns (Pl. I, fig. 7). In such regions, which are devoid of pore canals, it seems unlikely that the gland cells have any cytoplasmic connexions with the cuticle.

Previous work (Lees, 1947) has shown that in *Ornithodoros* the epicuticle of the general body cuticle is itself made up of successive thin layers of cuticulin, polyphenols, wax, and cement. Whether the pore canals penetrate the cuticulin layer, as in *Rhodnius* (Wigglesworth, 1947), is not known. If this is the case, their free ends are presumably covered over by the wax layer. The thin cement layer in turn forms an external protective covering over the wax. Now, once the cement has been laid down, no further deposits of wax can be secreted by the pore canals unless both cement and wax are removed (by abrasion, for example). Yet, as we have seen, the production of wax over Gén 's organ takes place freely at intervals throughout the life of the egg-laying female. It is, therefore, important to know whether the cement layer extends on to the organ.

The presence of cement on the general body cuticle can be demonstrated by extracting the whole tick with cold chloroform for 30 minutes and staining

n 5 per cent. ammoniacal silver nitrate. The cement is insoluble in cold chloroform and protects the underlying wax from solution. Therefore the polyphenols beneath the wax layer cannot reduce the silver reagent where cement is present and these areas remain unstained. Since the epicuticle of Gén  s organ also contains a polyphenol layer (covered in this case by the wax secreted by the organ itself) this method can also be used here for mapping the distribution of cement.

If a tick, with G  n  s organ everted, is immersed in ammoniacal silver without previous extraction, there is no staining of the cuticle, showing that the wax layer is complete (Pl. I, fig. 5). On the other hand, if the tick is



TEXT-FIG. 6. G  n  s organ in *Ornitodoros moubata* showing the area covered by the cement layer (A), the distribution of the pore canals (B), and the probable site of wax secretion (C).

previously extracted for 30 minutes in cold chloroform, the cuticle round the base of the horns, and the horns themselves, stain intensely (Pl. I, fig. 6); nevertheless, like the general body cuticle, most of the stalk still fails to stain. This shows that the cement layer extends on to G  n  s organ as far only as the base of the horns (Text-fig. 6A). The line of demarcation between the staining and non-staining areas is very sharp and corresponds with a line drawn round the circumference of the stalk which is well to the proximal side of a similar line marking the point of detachment of the epidermis from the cuticle. This result, which is of particular significance, was checked on sections of organs previously stained with silver (Pl. I, figs. 8, 9). There is a zone of varying width running round the circumference of the stalk between the bases of the horns where pore canals are present and cement absent (Text-fig. 6 C). In view of the marked chemical dissimilarity of the precursor from the horns and the wax from the surface of the cuticle (see p. 313), there seems no doubt that the former is first subjected to transformations by the living cells. If such is the case the wax must be secreted through those areas of the cuticle provided with pore canals (which contain, presumably, cytoplasmic processes extending from the living cells) and not through the non-living cuticle of the horns which is devoid of pore canals. According to this

view, the area shown diagrammatically in Text-fig. 6C also represents the site of the secretion of wax.

PROPERTIES OF THE *ORNITHODORUS* WAXES

The waterproofing waxes examined by us have been derived from the following sources. The egg wax was obtained in its natural state from deposits occurring on the outside of Gén 's organ; and small quantities of this wax were also extracted from vacated egg-shells. In addition, we have made some comparisons of the properties of the egg and cuticular waxes. The latter may be obtained in natural form from living ticks which have regenerated large quantities of this material after their cuticles have been thoroughly abraded with alumina dust (Lees, 1947), and the cuticular wax may also be extracted from cast nymphal skins.

Properties of the Natural Waxes

The colourless transparent wax removed from Gén 's organ has a somewhat syrupy consistency at 25° C. Small filaments of the wax, when warmed in a melting-point capillary, undergo rather indefinite optical changes between 36° and 46° C. and melt at 50–54° C. (Table 5). In view of the fact that the

TABLE 5. *Temperatures at which the natural waterproofing waxes of O. moubata, and the materials obtained from hot chloroform extracts, show optical changes*

<i>Material and source</i>	<i>Transitional changes ° C.</i>	<i>Melting-point, ° C.</i>
Natural wax; Gén�'s organ . . .	36, 40, 46	50–54
White wax; egg-shell extract . . .	56, 62, 72	Above 100
Yellow grease; egg-shell extract . . .	Indefinite	..
Total egg-shell extract . . .	48	57
Natural regenerated wax; adult cuticle .	38, 53	65
White wax; cuticle extract . . .	75, 90	107
Yellow grease; cuticle extract . . .	Indefinite	..
Total cuticular extract . . .	70, 84	89

critical temperature of the egg is about 45° C. (p. 302), it is interesting to recall that Beament (1945) has found that increased evaporation from the cuticle of insects is associated more closely with changes in the optical properties of the wax, which occur below the melting-point, than with the melting-point itself.

The egg wax appears brilliantly luminous when viewed in polarized light and, as with other polycrystalline lipoids, exhibits no position of extinction between crossed nicols. It dissolves instantly in cold or hot chloroform or xylene but is insoluble in alcohol. Lumps of wax stain deeply with sudan black B but not with sudan III. The protein colour tests (xanthoproteic, Sakaguchi, ninhydrin, and Millon reactions) are all negative. The significance of this latter observation is discussed on p. 329.

The wax recovered from the surface of the cuticle after abrasion has entirely different physical properties. At 25° C. it is a hard white crystalline solid which shows no tendency to 'creep' along surfaces. As judged by the optical properties, transitional changes occur at 38° and 53° C. and the wax melts at 65° C. The critical temperature of the cuticle, as we have previously noted, is approximately 62° C. (Table 4).

Properties of the Extracted Lipoids

The waterproofing substances from the cuticle were obtained by extracting the exuvia with chloroform. The cast skins were washed several times in cold water and dried in a desiccator. After repeated extraction in boiling chloroform under a reflux condenser, the solution was filtered through a hot-jacket funnel, evaporated to dryness at room temperature, and the total residue weighed.

The extracted material is not homogeneous, for two lipoids—a white waxy solid and a soft yellow grease—separate partially in the evaporating dish. Complete separation can be effected by treating the residue with acetone, in which only the grease is soluble. The white wax and the yellow grease are present in the total chloroform extract in the ratio of about 6:1 by weight.

The white crystalline wax has the following properties. Although the purified substance only melts to a clear liquid at 107° C., it undergoes optical changes, such as clearing, darkening, and loss of crystalline form, at lower temperatures (Table 5). These changes may be associated with crystalline transformations. The wax is soluble in boiling chloroform or benzene, and also in hot pyridine, but is sparingly soluble in the cold. It is insoluble in water, hot or cold alcohol, and hot or cold acetone. There are some indications that the material is capable of forming an oriented layer. Thus, if a crystal of the wax is dropped on to the surface of boiling water it forms a fine film which may be obtained by dipping a glass-slide through the surface; and this film has similar properties to the crystalline material save that it is now wetted by 50 per cent. alcohol. The white wax is not stained by either of the sudan stains, or by Ciba's B.Z.L., nor is it darkened by osmium tetroxide.

The second component, the yellow grease, is readily soluble in cold lipophilic liquids, including acetone and absolute alcohol; it stains with sudan III and with sudan black B and darkens with osmium tetroxide. The optical changes with temperature are indefinite.

Hot chloroform extracts of egg-shells (separated first from the larval exuvia) also yielded two fractions comprising white wax and yellow grease in approximately the same proportions as in the cuticle extracts. Their properties also appear to be very similar (for temperature changes see Table 5).

It would appear that the white wax may be a mixture of very long chain paraffins, acids and esters, while the grease may be composed of much shorter molecules, with unsaturated bonds along the chain.¹ In the natural waxes—the regenerated material from the cuticle or the wax from Géné's organ—the

¹ Private communication from Prof. A. C. Chibnall, F.R.S.

two lipoids seem to be present in some stable form of association, although this may not necessarily be of a strong chemical nature. This state does not appear to be automatically reconstituted when the two substances have been extracted with lipid solvents. Nevertheless it is noteworthy that when the two components derived from the cuticular extracts are intimately mixed, the resulting melting-point of the mixture is considerably lower than that of the purified white wax and approaches the melting-point of the natural regenerated cuticular wax (Table 5).

The Spreading Properties of the Egg Wax

Spreading on the Egg. Since a comparatively small part of the egg-shell appears to come in contact with Gén  's organ during the normal oviposition movements (see p. 295), the newly secreted wax must be capable of spreading over the surface of the shell. The spreading properties of the wax were therefore tested in *O. moubata*. Unwaterproofed eggs were obtained as usual by allowing ticks with Gén  's organ covered to deposit egg-clusters in damp air; and the wax was obtained from ticks kept continuously at 15   C., organs bearing massive deposits of wax being selected for use. Each egg was removed individually from the egg-mass with forceps, brought into contact with the wax on the everted organ, and manipulated so that the wax was thoroughly spread over the required surface. Sometimes the eggs of a batch were completely smeared with wax; sometimes, in order to test the spreading powers, approximately half the surface was smeared. After treatment the eggs were placed on damp filter-paper for 2 hours and were then exposed in a watch-glass to dry air at the same temperature. The control eggs, which remained unwaxed, were always drawn from the same egg-mass.

TABLE 6. *The effect of smearing unwaterproofed eggs of O. moubata with wax taken from Gén  's organ of the same species*

The rate of shrivelling was observed in dry air

Female no.	Treatment	Temperature, �� C.	No. of eggs	No. of eggs shrivelled after		
				30 min.	4 hrs.	24 hrs.
1	{ None Wholly smeared	20	20	20	..	..
			20	6	11	16
2	{ None Half-smeared	25	20	18	20	..
			20	0	0	3
3	{ None Half-smeared	30	20	13	20	..
			20	0	0	0
4	{ None Half-smeared	19	20	19	20	..
			20	20	..	..
5	{ None Half-smeared	19	20	2	4	10
			20	2	2	4

The results, which are given in Table 6, show that the rate of shrivelling is considerably reduced when the eggs are completely smeared with wax. Nevertheless, in spite of the fact that the amount of wax transferred to each

egg is probably greatly in excess of the normal quantity, and that a greater surface area of the shell is brought into contact with the wax than during the natural manipulation by Gén 's organ, the final degree of impermeability is always inferior to that of the egg when waterproofed naturally.

Results with half-smear 'd eggs proved to be very variable. In some egg batches (e.g. females 2 and 3) the permeability was greatly reduced, indicating that considerable spreading of the wax had taken place. In others (e.g. female 4) the eggs shrivelled as rapidly as the controls, thus providing no evidence of effective spread. No certain correlation between temperature and the rate or efficiency of waterproofing could be established. But the waterproofing effect is usually more pronounced if eggs are selected whose shells, even before the application of wax, are relatively impermeable (e.g. female 5, Table 6).

No evidence of spread was obtained with smaller areas of contact. A number of eggs, each with a large lump of wax adhering at one point, shrivelled as rapidly as the controls at an incubation temperature of 30  C. The limited spreading powers of the wax are also shown by its inability to spread effectively from egg to egg. A number of unwaterproofed eggs were, for example, placed in contact with normal waterproofed eggs or were thrust into the centre of a normal egg-mass. Yet these always shrivelled on subsequent exposure to dry air. If a female tick is interrupted in the process of oviposition, G 'n 's organ never bears any large wax deposits although there is always a film of wax bounding the epicuticle (as can be shown by the failure of the epicuticle to stain with silver from the outside). This film of wax is also incapable of limiting evaporation from unwaterproofed eggs if these are brought into intimate contact with the organ. It would appear, therefore, that the production of wax by G 'n 's organ may, to some extent, be co-ordinated with the delivery of the egg by the vaginal 'ovipositor'.

An additional factor influencing the spread of wax is probably the smoothness of the substratum. It was noted that waterproofing was invariably poor if any fungal hyphae were growing on the surface of the shell.

It is possible also to reduce the permeability of *Ixodes* eggs by applying the egg wax of *Ornithodoros*. Eggs laid without G 'n 's organ (and therefore already partially covered with *Ixodes* wax) were completely smeared with wax on an everted organ of *Ornithodoros*. Water loss was then found to be slightly less than from normal *Ixodes* eggs. The following are examples of the results obtained with batches of 20 eggs laid by the same tick. In a normal batch, laid with G 'n 's organ, 4 eggs had shrivelled after 4 hours, and 20 after 24 hours, in dry air at 30  C. In a second batch, laid with G 'n 's organ covered, every egg was completely dried out within 4 hours. In a third, laid without G 'n 's organ, but wholly smeared with *Ornithodoros* wax, no eggs had shrivelled after 4 hours and only 15 after 24 hours. The effectiveness of the waterproofing is, however, hardly comparable with that exhibited by the normal *Ornithodoros* egg.

Spreading on Membranes. Beament (1945) has shown that extracted insect cuticular waxes may be deposited on prepared membranes from chloroform

solutions. Provided that the surface film is continuous the membrane then reproduces many of the physical properties (e.g. critical temperatures) of the normal insect cuticle from which the wax was obtained. Experiments were carried out to determine whether natural or extracted waxes of *Ornithodoros* could also be used in a similar manner. The membranes consisted of lipoid-free butterfly wings, chloroform-extracted wings of *Rhodnius prolixus*, and large wax-free nymphal skins of *O. moubata*. These were mounted in holders of the type described by Beament (loc. cit.); the surface area exposed was adjusted so as to range from 3 to 50 mm.². The sources of the waxes and the methods of application were as follows: (i) Natural wax from Gén  s organ was smeared in the centre of the membrane. (ii) Wax from Gén  s organ was deposited on the membrane from cold chloroform. (iii) The total chloroform extract from egg-shells, the acetone-soluble fraction (yellow grease), and the chloroform-soluble fraction (white wax) were deposited separately from cold and hot chloroform. (iv) The same procedures were also carried out with regenerated cuticular wax and with the cuticle extract.

Because of several initial failures, each waxed membrane was subsequently heated in an oven at different constant temperatures which varied from 30   to 70   C. It was hoped that heat would assist spreading in an uneven deposit of wax and promote an alinement of the wax molecules more consistent with impermeability. The steady water loss from each membrane was recorded at room temperature before and after heat treatment.

In none of the membranes could any reduction in permeability be detected, with the exception of the following. A lipoid-free *Ornithodoros* nymphal skin, 3 mm. in diameter (and therefore about three times the surface area of an *Ornithodoros* egg) had an initial permeability at 20   C. of 20 mg. sq.cm. hr. After smearing it with the natural wax from Gén  s organ and heating to 50   C. the rate of transpiration at 20   C. fell to 5 mg. sq.cm. hr. Nevertheless, this membrane was still too permeable for testing the critical temperature. It seems clear from these results that the natural egg wax must spread relatively slowly, even at high temperatures. And chloroform appears to have the effect of separating the wax system into two components neither of which can be redeposited on a membrane in the form of a waterproofing layer.

Spreading on Water. The spreading pressures of substances on water may be compared in the apparatus described by Adam (1945). Water is introduced into a large funnel immersed in a water bath and is flushed through until the surface is clean. This is then covered with a fine film of lycopodium powder which will indicate changes in the surface when any foreign material is added.

If Gén  s organ is everted and applied to the centre of the prepared water surface, the powder is displaced slowly and a film of wax spreads from the organ over a small area. We have noted that the wax is not very mobile at room temperatures; the secretion spreads more rapidly if the temperature of the water surface is raised, but it appears to cover only the same small area.

If the eggs of *O. moubata* are dropped on to the prepared water surface, slow spreading over a limited area takes place at all temperatures between

20° and 60° C. This phenomenon was obtained with newly laid eggs and with those about to hatch. It is obvious that the waterproofing lipoid is not completely bound or otherwise immobilized on the shell surface.

Material spreads much more rapidly from the surface of eggs of *I. ricinus* at room temperature and extremely rapidly at higher temperatures, but the waxy film again fails to cover a very large area. By comparison with the spreading powers of the waterproofing grease from the cockroach cuticle (Beament, 1945), the surface activity of the tick waxes is slight. Such spreading powers as the egg waxes possess seem to be associated with the presence of the acetone-soluble material (the yellow grease) which spreads actively. The white wax is devoid of spreading properties.

The Amount of Wax present on the Egg

The average weight of waterproofing material on the egg can be derived from a knowledge of the weight of extracted material and the total number of egg-shells extracted. Assuming the relative density to be 0.96 g./c.c. (Lewkowitsch and Warburton, 1921), the average volume of wax covering these eggs can be found. The approximate surface area of the egg may be arrived at from camera lucida drawings and geometric considerations. The thickness of the extracted materials, if spread evenly over the surface of the egg, can then be calculated.

The thickness of the wax layer in the *Ornithodoros* egg was found to be approximately 0.47 μ and in the *Ixodes* egg 1.76 μ . On the other hand, the thickness of the cuticular wax layer in the nymph of *Ornithodoros* is no more than 0.29 μ .

It will be noted that in both species of ticks the waterproof covering of the egg is considerably thicker than the wax layer encountered on the inside of the *Rhodnius* egg-shell (Beament, 1946b); and it is also of greater thickness than the waterproofing layers on the cuticles of several insects (Beament, 1945), and of the nymphal tick itself. The calculation made above assumes an even thickness of wax on the egg. In fact, visual inspection shows that this is by no means always true, for eggs may often be seen in the egg-masses of *Ornithodoros* which have definite mounds of wax adhering at one or more points. Nevertheless, the figures may be taken to indicate that each egg receives a slight excess of wax.

THE NATURE OF THE CONTENTS OF GÉNÉ'S ORGAN

The nature of the glandular secretion of Géné's organ is of considerable interest as it is evident that the secretion is associated in some way with the production of wax on the outside of the organ and may in fact be the wax precursor.

The contents of the horns, a clear refractile liquid, can be examined *in situ* with the 1/12th oil immersion objective. Apart from pieces of tissue (derived from fragmentation of the gland), which are abundant in some organs but completely absent in others, no particles or droplets are visible within the limits of resolution.

It is possible to collect small quantities of the liquid by applying a fine ligature of silk thread at the base of each horn and another stouter ligature round the base of Géné's organ, then cutting it free and pricking each horn



TEXT-FIG. 7. The method of ligaturing the horns of Géné's organ in *Ornithodoros moubata*.

separately on to a glass slide. The method is illustrated in Text-fig. 7. By careful selection of the ligatured organs it is possible to obtain the secretion virtually free of organic debris from the gland.

The liquid from the horns can be shown to contain water by pricking the ligatured horn on to a small crystal of anhydrous cobalt chloride in a dry atmosphere. The crystal instantly turns red. If the water is allowed to evaporate after pricking on to a glass slide, a speck of solid material is left.

This is perfectly transparent and is sometimes slightly brittle, but has a waxy feel when scratched with a needle. On adding droplets of warm or cold water the material redissolves instantly. Xylene and chloroform, which are excellent solvents for the wax deposits on the outer surface of the horns (p. 308), fail even to wet it.

Protein or protein derivatives are present in the contents of the horns. The following tests, which are summarized in Table 7, were performed on specks

TABLE 7. *Some properties of the material isolated from the gland of Géné's organ in O. moubata*

Test	Contents	Observation
Water	Solid	Dissolves instantly
Arginine reaction	Aqueous solution	Strongly positive
Ninhydrin	" "	Positive
Xanthoproteic reaction	" "	Negative
Aldehyde reaction	" "	"
Millon's reaction	" "	"
Ethyl alcohol	" "	White flocculant precipitate
Phosphotungstic acid	" "	" " "
Phosphomolybdic acid	" "	" " "
Picric acid	" "	Yellow precipitate
Aluminium chloride	" "	Solution remains clear
Sodium citrate	" "	" " "
Adjust to pH 8 or to pH 3	" "	" " "
Sudan III and sudan black B	Solid	No staining
Heat	" "	Does not melt at 100° C.
Crossed nicols	" "	Faintly luminous

of the material redissolved in distilled water. The ninhydrin reaction is positive indicating the presence of amino groups. The Sakaguchi reaction for arginine, which was performed by adding successive droplets of 5 per cent.

NaOH, 1 per cent. alcoholic α -naphthol, and 10 per cent. sodium hypochlorite, is invariably strongly positive. Other colour reactions, including the sulphur reaction for cystine, the aldehyde reaction for tryptophane, the Millon and xanthoproteic reactions, are negative. The material must, therefore, be poor in tyrosine and tryptophane.

The protein in the horn contents is rapidly coagulated by the addition of ethyl alcohol forming a flocculent white precipitate. It is not precipitated by concentrated nitric acid in which the solid dissolves to yield a clear solution; neither is it readily heat-coagulable. If the solid is warmed on a slide at 100° C. for 1 hour, it immediately redissolves on the addition of water and this also occurs after warming in the presence of a droplet of 1 per cent. acetic acid. With stronger heating over a flame the solid material is gradually coagulated and fails to redissolve completely in water. It does not melt.

The protein is readily precipitated in aqueous solution by alkaloidal reagents such as picric, phosphotungstic, phosphomolybdic, and trichloroacetic acids and fails to redissolve in water.

We seem to be dealing here with a protein rich in di-amino-acids. The high arginine content, solubility in water, and the resistance to coagulation by heat suggest some affinities with the protamines or histones. The reactions detailed above could not all be ascribed to the presence of free amino-acids, peptones, or proteoses (which might conceivably be present as emulsifying agents).

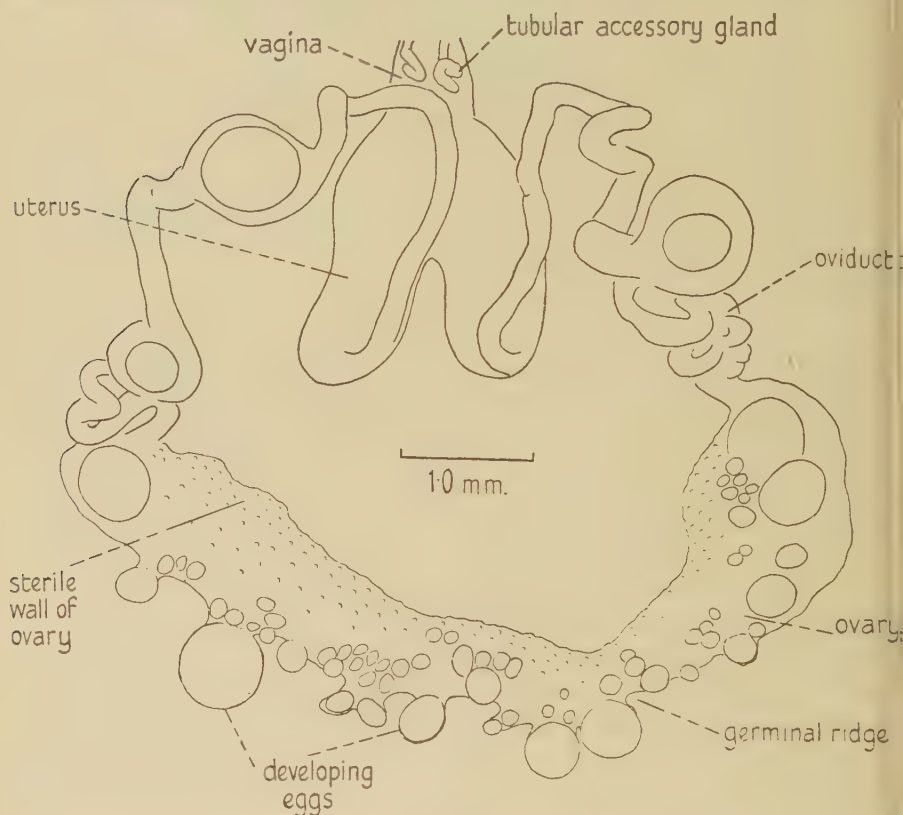
With the minute quantities of material available for tests, it has not been possible to isolate from the contents of the horn any material whose identity with the wax could be proved beyond doubt. Two observations do, nevertheless, lend some support to the view that the horns contain the wax precursor. First, a speck of the solid material treated with 5 per cent. trichloroacetic acid becomes difficult to wet with water, the surface developing at the same time a glistening waxy appearance. However, although the coagulum is now readily wetted by chloroform little appreciable solution takes place. Secondly, the appearance in polarized light also affords some slight evidence of the presence of long chain lipoids. A small patch of material from the horns is isotropic when viewed normal to the surface but particles scraped up on a needle are decidedly birefringent in all quadrants. The material is therefore polycrystalline, as the wax itself is (p. 308).

Certain facts suggest that the wax is not present in the horns as a simple emulsion. For example, the horn contents are not readily precipitated by changes in the reaction of the medium. If the solution is adjusted to pH 8 or pH 3 by adding, respectively, droplets of borate or phthalate buffer, no trace of milkiness develops. Further, the solution remains perfectly translucent in the presence of excess polyvalent ions (e.g. calcium, aluminium, citrate).

The horn contents have no powers of emulsifying waxes in bulk. The absence of any effective agent can be shown by pricking a ligatured horn on to a small lump of wax removed from the outer surface of Géné's organ. No visible emulsification takes place and the watery contents of the horn fail even to wet and mix with the wax.

THE MORPHOLOGY OF THE FEMALE GENITAL SYSTEM IN TICKS

Several detailed accounts of the morphology and histology of the genital system are available. Among Argasidae we have descriptions for *Ornithodoros savignyi* (Christophers, 1906) and *Argas persicus* (Robinson and Davidson, 1914); and among Ixodidae for *Ixodes ricinus* (Samson, 1909) and *Dermacentor andersoni* (Douglas, 1943). Some of the salient features will be recapitulated.



TEXT-FIG. 8. Female genital system in *Ornithodoros moubata*.

below. Our inquiry has, however, been directed principally towards two specific questions. In the first place, a rather more detailed treatment of the development of the ova in *O. moubata* has been a necessary preliminary for an extended examination of the structure and properties of the egg-shell. And secondly, the genital tract in *I. ricinus* has been re-examined in an attempt to discover the site of application of wax to the egg, for, as we have shown, a waxy covering is already present on the egg before it is received by Gén 's organ.

The Genital Tract and Ovary in O. moubata

The genital system of an egg-laying female is shown in Text-fig. 8. There is, as in all ticks, a single garland-like ovary which passes at each end into the

long coiled oviducts. The posterior wall of the ovary is studded with developing ova of all sizes, but the anterior wall is sterile and consists of undifferentiated cells only. This type of tubular ovary with a parietal germarium occurs throughout the Arachnida and may represent an archaic feature of their organization. The tick ovary, indeed, closely resembles that of the Onychophora (Manton, 1938).

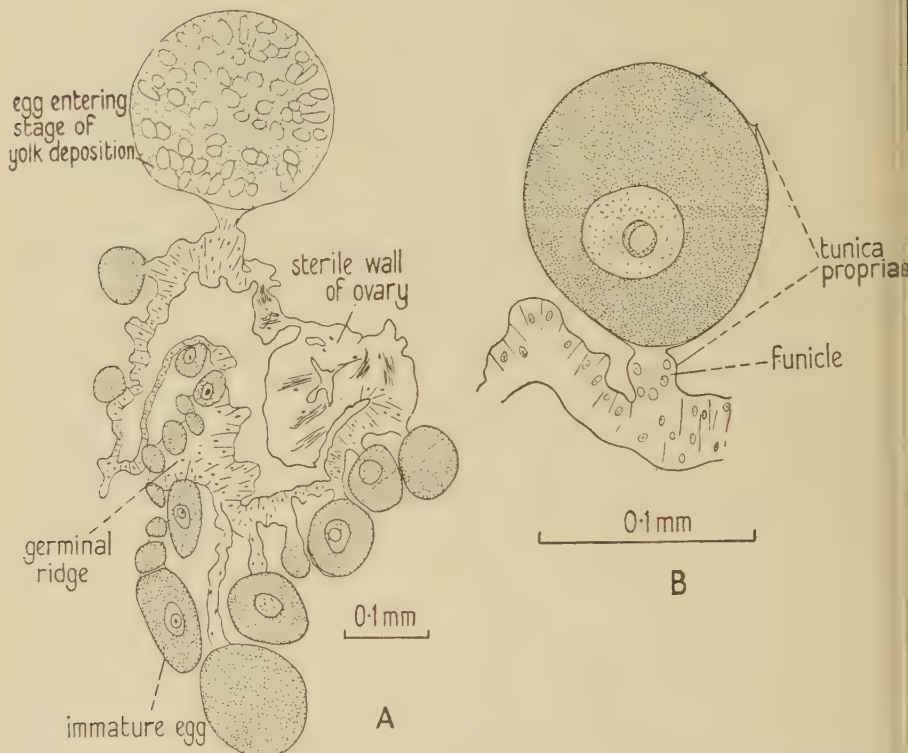
The oviducts pass into the large sac-like uterus from which arises the short vagina leading to the genital aperture. Robinson and Davidson (1914) distinguish two sections in the vagina, a posterior or cervical region, provided with a thick investment of muscles, and a short vestibular region without circular muscles. Two small tubular accessory glands enter the vagina near their junction.

The general course of development of the eggs appears to be as follows. The ova arise from small undifferentiated cells lying in the germinal ridge. This part of the ovary is itself invested externally by a fine hyaline non-chitinous membrane, the tunica propria. As the egg grows in size it begins to stretch this membrane and projects outwards from the surface of the ovary (Text-fig. 9A). By the time a diameter of 100 μ has been attained the egg has acquired a stalk or funicle whose walls are made up of a single layer of undifferentiated epithelial cells (Text-fig. 9B). These cells stain blue in Mallory, whereas the cytoplasm of the immature egg acquires a strong affinity for acid fuchsin. Having attained a diameter of about 500 μ the egg is finally ovulated into the lumen of the ovary. This process appears never to have been observed, but there is little doubt that, as Christophers (1906) implies, the continuous growth of the egg must impose an increasing strain on the elastic tunica propria so that eventually pressure forces apart the cells of the funicle and the egg passes through.

The appearance of the shell can first be detected when the egg is 25 μ or less in diameter. According to Nordenskiöld (1909) the shell in *I. ricinus* is first laid down in the form of adjacent granules which coalesce to form a continuous membrane, the last region of fusion being in the region of the funicle. This author held therefore that the funicle played some part in secreting the shell; and also that nutritive material for the developing egg is drawn from the lumen of the ovary via the funicle, as well as from the haemolymph through the tunica propria. In our sections of the ovary in *Ornithodoros* the shell first appears as an exceedingly tenuous pellicle which is continuous over the funicular region, as elsewhere. It is to be doubted whether the funicle plays any part in secreting the shell for the two are not firmly attached to one another. This can be shown if the ovary is dissected in saline and the tunica propria is slightly torn with a needle. Developing ova of all sizes then roll out freely into the dissecting-dish. Yet the shell clearly increases both in surface area and in thickness as the ovum grows in size within the tunica propria. It is apparent from this that the shell must be secreted by the oocyte itself. Among the insects the term 'chorion' is best reserved for those parts of the shell secreted by the follicle cells (Beament, 1946a). We shall not therefore apply this term to the tick egg-shell.

As the egg passes down the ovary into the oviduct it absorbs water, finally reaching a diameter of about $800\ \mu$. The eggs are stored for a short while in the uterus before being laid.

The outer surface of the tick egg is completely smooth and featureless: there is no micropile. Robinson and Davidson (1914), finding sperms only as



TEXT-FIG. 9. The structure of the ovary in *Ornithodoros moubata*: A, transverse section through a mature ovary; B, section through an immature egg developing in the germinal ridge.

high as the upper oviduct in *A. persicus*, suggested incorrectly that the shell was laid down by the oviduct shortly after fertilization. But we have seen that in *O. moubata* the shell rudiment is acquired very early in development. How then is the egg fertilized?

In this species, as in other Argasidae, the uterus also serves as a receptaculum seminis, often containing as many as a dozen spermatophores. As these are ruptured, the sperms, which are relatively gigantic objects some $400\ \mu$ in length, make their way up the oviducts, presumably by means of the slow gliding movements of which they are capable. The genital tracts of fertilized ticks, which were dissected in Ringer and examined with a $1/6$ th objective, often contained isolated sperms in the lumen of the ovary as well as large clusters of sperms in the upper oviducts. There would appear to be no

escape from the conclusion that the oocyte is fertilized *in situ* within the germinal ridge before the rudiments of the shell are laid down at the beginning of the growth phase.¹ It is of considerable interest in this connexion that Christophers (1906) has figured a developing egg in *O. savignyi* only about one-quarter grown and yet containing the clearly recognizable remains of a spermatozoon. In spite of careful search, however, we have found no similar traces within the eggs of *O. moubata* at any developmental stage. Nevertheless, in view of the resistant nature of the egg-shell (p. 322), it is highly improbable that sperms could enter the egg after the deposition of the shell has begun. Uterine eggs are usually surrounded by spermatozoa but they never penetrate the shell.

The Genital Tract in Ixodes ricinus

The ovary in this Ixodid is much longer than in *O. moubata* and contains greater numbers of developing eggs. On the other hand, the general course of ovulation and of egg development is probably very similar and need not be described here.

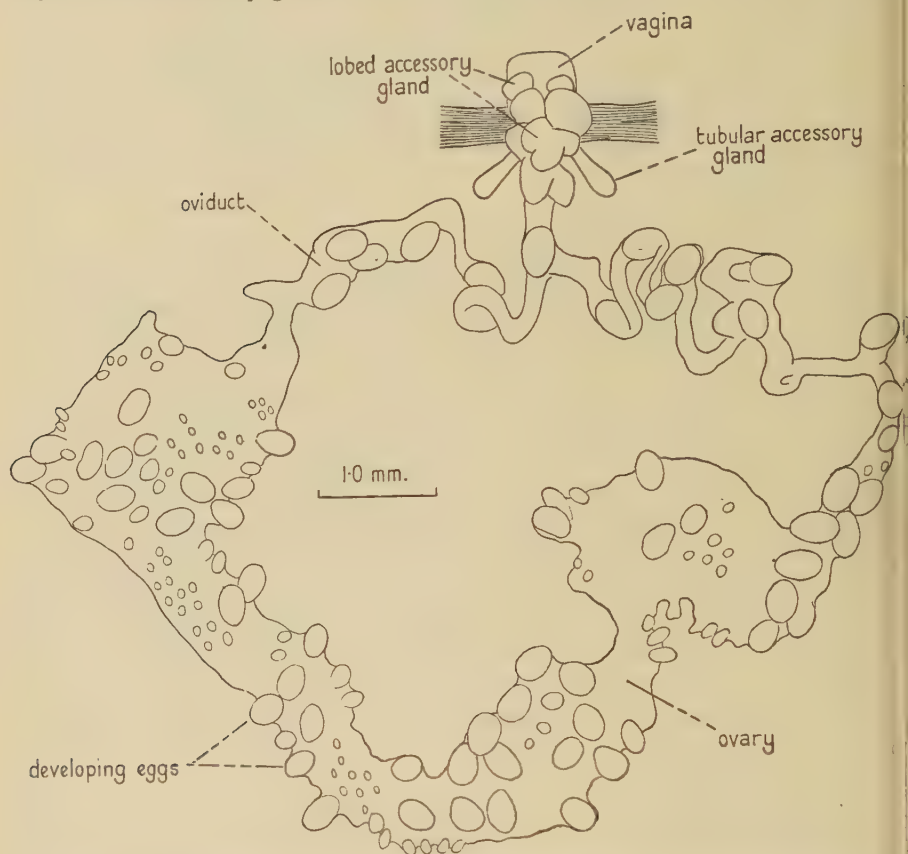
There are, however, important morphological differences in the distal regions of the genital tract. There is no uterus in *I. ricinus* and the two oviducts join to form a short common oviduct leading directly to the vagina (Text-fig. 10). Further, there are two types of accessory glands: short, club-shaped or tubular glands, probably homologous with the tubular glands in *Ornithodoros*, and a larger gland whose numerous irregular lobes are disposed on the dorsal and lateral sides of the vestibular portion of the vagina. The latter is without a homologue in *Ornithodoros*. Douglas (1943) refers to this lobed accessory gland in *Dermacentor andersoni* as a receptaculum seminis and regards it as comparable with the uterus in Argasidae—a term which, in his opinion, is a misnomer. In *Ixodes*, however, the lobed accessory glands never contain spermatophores; on the contrary, these seem to be passed directly into the common oviduct.

The general arrangement of these organs is shown in the sagittal section (Text-fig. 11). The cervical and vestibular regions of the vagina, with the tubular accessory glands opening near their point of junction, can again be distinguished. The entire vagina is lined by thin cuticle which is continuous with the general body integument. Over the vestibular region, epicuticle and a thin layer of endocuticle is present; it is highly folded and in places bears minute recurved spines. In the cervix the intima is reduced to a tenuous layer of epicuticle which closely follows the contours of the vaginal cells. The latter are closely invested with a deep layer of circular muscle-fibres which, by their contraction, are probably responsible for the prolapse of the vagina during oviposition (Pl. I, fig. 12).

We have shown that the eggs dissected from the oviducts are completely unwaterproofed but that they acquire an incomplete layer of wax on the

¹ The precocious uptake of sperms by the young oocyte is described in *Peripatopsis* by Manton (1938), but here the function is stated to be that of providing nutriment for the growth of the ovum.

surface of the shell during their passage down the vagina. The source of this wax may therefore be (i) the cells lining the cervical region of the vagina, (ii) the tubular accessory glands, or (iii) the lobed accessory glands. Their histology affords convincing evidence of the close affinity of one of the organs—the lobed accessory glands—with Gén 's organ.



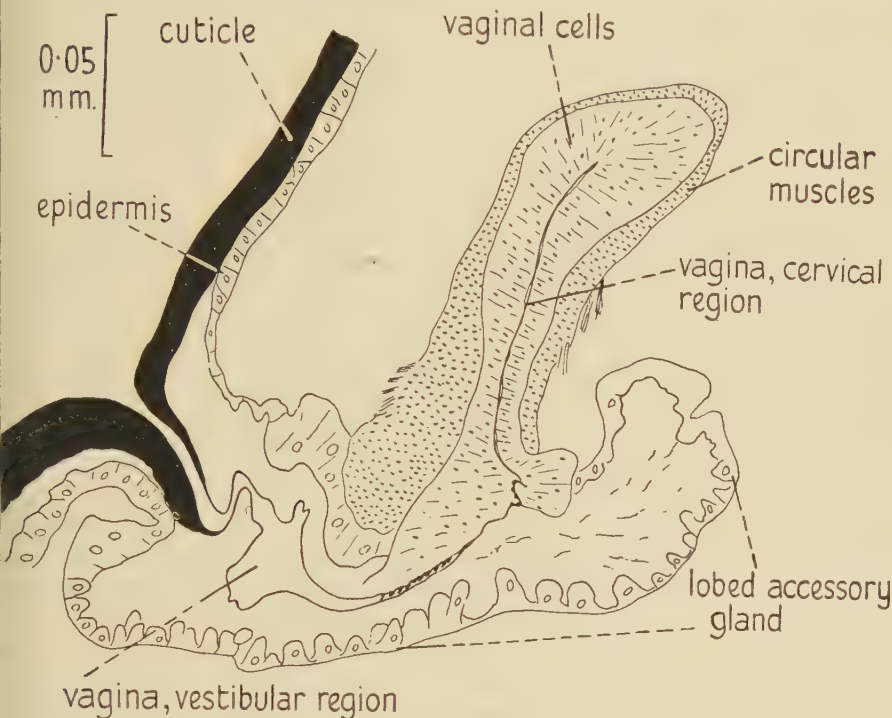
TEXT-FIG. 10. Female genital system in *Ixodes ricinus* towards the close of oviposition.

A section through the wall of one lobe of Gén 's organ is shown in Pl. II, fig. 10. The histological appearance is completely different from that of the corresponding organ in *O. moubata*. The gland-cells are very large by comparison, are often widely spaced from each other, and are irregular or polygonal in shape. The cytoplasm is dense and deeply staining, the nuclei are large, and there is a well-developed 'honeycomb' border. Not infrequently an irregular meshwork of fibres extends from the cell margin into the gland lumen.

The tightly packed columnar epithelial cells lining the cervix have poorly defined cell boundaries and small nuclei disposed at different levels in the cell (Pl. I, fig. 12). As already mentioned, the cuticular intima maintains close contact with the free borders of the cells.

The tubular accessory glands are built up of wedge-shaped cells bordering a narrow central lumen (Pl. I, fig. 13). Their cytoplasm usually contains large droplets of colloid which stain a pale grey with iron haematoxylin. The lumen appears to open freely into the genital tract.

On the other hand, the histological appearance of the lobed accessory gland (Pl. I, fig. 11) and the gland of Gén 's organ is so similar that the two cannot



TEXT-FIG. 11. Median sagittal section through the female genital tract in *Ixodes ricinus*.

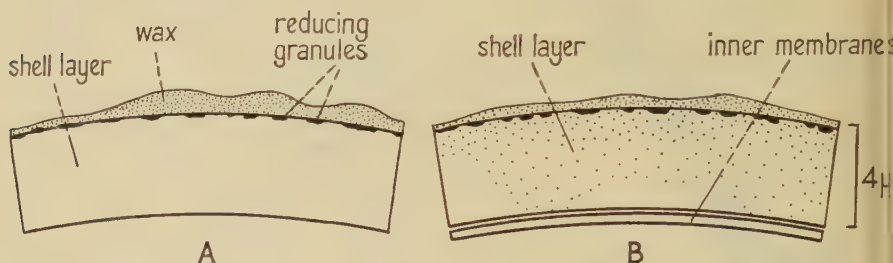
asily be distinguished unless the arrangement of the lobes is followed carefully in serial sections. Probably, therefore, the former is responsible for secreting the wax. It may be noted that this gland bears the same relation to the vaginal cuticle as does the gland of Gén 's organ to the cuticle of the horns, for the lumen of the accessory gland is partitioned off from the genital tract by the cuticular lining of the vestibule, which is in contact with the cells only near the margins of the gland. The accessory gland—also like Gén 's organ—represents an intucked region of the epidermis which has become specialized for a particular glandular function.

An additional point of interest is the absence of a homologous organ in *D. moubata*. In this species, as we have seen, there is no evidence that waxes are secreted on to the egg during its passage down the vagina.

THE STRUCTURE AND CHEMISTRY OF THE EGG-SHELL IN *O. MOUBATA*

Three distinct layers can be distinguished in the shell of the newly laid egg: (i) an inner 'shell layer'; (ii) an incomplete layer of granules with reducing properties; and (iii) the outer covering of wax which is applied by Génée's organ (Text-fig. 12A). A fourth layer, the 'inner membrane' (iv), is secreted after the egg has been incubated for 2–3 days (Text-fig. 12A, B). The properties of these layers may now be outlined; those of the wax have already been described.

The Shell Layer. One-day-old eggs were punctured, freed from yolk in distilled water, dried, and extracted in boiling chloroform to remove soluble



TEXT-FIG. 12. Diagram illustrating the structure of the egg-shell in *Ornithodoros moubata*. A, 0–1 days after laying. B, After incubation for 6 days at 25° C.

lipoids. The observations on this material were repeated on shells of eggs dissected from the ovaries at various stages of growth and on eggs which had been laid without establishing contact with Génée's organ. The latter are, of course, free from wax and need not be extracted first in chloroform.

The lipid-free shell layer is a uniform membrane with striking elastic properties, smooth on its outer and inner surfaces and 3–4 μ in thickness. It is colourless, the tint of the egg depending entirely on the pigment in the yolk. There are no pore canals.

The shell layer contains no chitin but it gives a strong xanthoproteic reaction and turns pink when heated with Millon's reagent. The ninhydrin reaction is negative and it fails to stain with cold and hot *p*-benzoquinone. The shell material stains deeply and rapidly with basic and acid fuchsin, borax carmine, haematoxylin, and picric acid. It fails to take up colour from alcoholic solutions of sudan III or sudan black B, nor does it darken with osmium tetroxide.

The shell membrane is insoluble in cold concentrated nitric acid, but dissolves on heating, leaving a granular residue; no oil is released. It is partially soluble in cold concentrated nitric acid saturated with potassium chlorate and on warming dissolves completely with the evolution of gas. The shell material dissolves readily in hot potash and is also rapidly broken down by 14 per cent. sodium hypochlorite.

This evidence shows that the shell layer is composed of a protein which, unlike the 'cuticulin' of *Rhodnius* epicuticle (Wigglesworth, 1947), does not incorporate a lipid.

The Granular Layer. If whole eggs, laid without Gén 's organ, are immersed for 15 minutes in 5 per cent. ammoniacal silver nitrate, the outer surface of the shell layer, after mounting, is seen to be covered with scattered granules and granule aggregates which have stained a deep reddish-brown (Pl. I, figs. 14, 15). The nature of these granules is uncertain for they are also stained by 1 per cent. silver nitrate (but not by silver nitrate in the presence of nitric acid). They appear on the shell layer shortly before the ovulation of the egg. Two further points are worthy of note. First, the shell layer has the appearance of a material which is partially tanned. Thus the shells of eggs newly ovulated into the lumen of the ovary are decidedly flabby and elastic, but they lose much of this elasticity and become much more rigid as they pass down the genital track into the uterus. Secondly, the reducing granules of the tick egg, although not identical with the polyphenol granules which constitute the substrate for the waterproofing layer in the *Rhodnius* egg-shell (Beament, 1946a), occupy the same functional position with respect to the wax layer.

The Inner Membrane. After incubation for 2–3 days at 25  C. a further layer, also secreted by the embryo, is added to the inner surface of the shell. It is easily demonstrated if empty egg-shells are treated with sodium hypochlorite solution: this dissolves the shell layer, leaving a tenuous transparent membrane which retains the same outline. The inner membrane is absent in the one-day egg.

The chemical properties of the inner membrane are mainly negative. It gives no definite colour with Millon's reagent and the xanthoproteic and ninhydrin reactions are negative. It does not contain chitin. The material is insoluble in cold and hot nitric acid but eventually soluble in hot nitric acid saturated with potassium chlorate. No oil is liberated when solution takes place. It is insoluble also in sodium hypochlorite and aqueous potash, but dissolves in fused potash. Water-soluble dyes such as borax carmine, basic or acid fuchsin fail to stain the membrane and it remains uncoloured after treatment with *p*-benzoquinone and ammoniacal silver nitrate.

The resistance of this material to attack by chemical agents therefore recalls the properties of the fertilization membrane of the *Rhodnius* egg (Beament, 1948a).

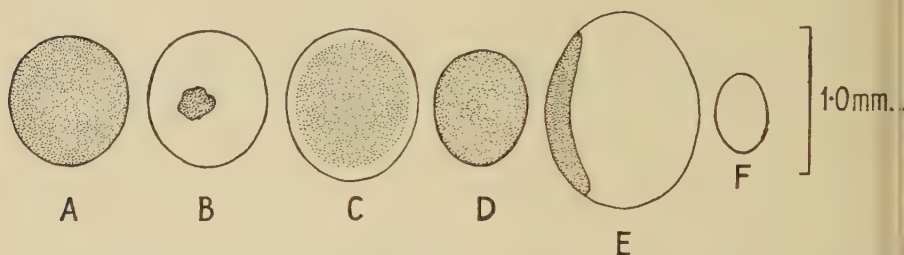
PERMEABILITY OF THE EGG-SHELL

Eggs which have received no wax from Gén 's organ are highly permeable to water and shrink rapidly in dry air (p. 296). As might be expected, unwaterproofed eggs of *O. moubata* also show notable swelling properties when immersed in distilled water. Developing ova taken from the germinal ridge of the ovary swell to nearly three times their normal diameter in 5 minutes before bursting (Text-fig. 13D, E); those from the uterus, because of their more rigid shells, swell more slowly, a hyaline zone appearing between

the yolk and the shell (Text-fig. 13A, C). Swelling may finally cease altogether.

We have already noted that water loss from the one-day egg, waterproofed in the normal manner by Gén  s organ, is enormously hastened by extraction in cold chloroform. This statement, however, does not necessarily hold good for eggs in a later stage of incubation. It has been found indeed that the effect on transpiration gradually diminishes as the period of incubation lengthens and that the eggs also develop increasing resistance to treatment with abrasive dusts. Some of the results obtained with eggs of known age are set out in Table 8.

After incubation for 3 or 4 days at 25   C., short extractions (1 min.) in cold chloroform, which undoubtedly dissolve all the wax from the surface of the



TEXT-FIG. 13 A-E. Eggs of *Ornithodoros moubata* after various treatments: A, normal egg; B, waterproofed egg extracted for 4 hrs. in cold chloroform; C, unwaterproofed egg from uterus after 30 min. in distilled water; D, egg dissected from germinal ridge; E, a similar egg after 5 min. in distilled water. F, egg of *Ixodes ricinus* in outline.

shell, leave the egg almost as resistant to desiccation as previously. Longer periods of extraction have a noticeable effect on older eggs, while the longest extraction (4 hrs.) completely destroys the waterproof properties, even in those eggs which are on the point of hatching. Penetration is particularly noticeable after 4 hours for chloroform gradually displaces the water in the egg and, without any accompanying change in the egg volume, forms a refractile zone round the shrivelled yolk (Text-fig. 13B). In a comparable manner, the one-day egg is highly sensitive to abrasion and shrivels immediately in dry air after it is rubbed with alumina dust or even if it is merely sprinkled with dust: whereas the older eggs again develop considerable resistance to this treatment, sometimes being seemingly unaffected by the most thorough abrasion (Table 8).

Two explanations of this phenomenon seem possible. On the one hand, the mobile wax applied by Gén  s organ to the outside of the shell may gradually sink in and impregnate the shell layer; it would then still be liable to extraction by prolonged treatments with chloroform but would be inaccessible to the influence of abrasive dusts. Or, alternatively, one could postulate the deposition of a secondary waterproofing layer by the embryo during the course of incubation—an event which occurs during the develop-

ment of the *Rhodnius* egg (Beament, 1948b). Indeed the inner membrane, which is secreted after 2–3 days of incubation, might possess the necessary waterproof properties. In order to resolve this question further evidence was sought regarding the permeability of the layers of the egg-shell both to water and to other substances.

TABLE 8. *The number of eggs, in batches of 10, that shrivelled in dry air at 25° C. after the following treatments: a, b, c, extraction in cold chloroform for 1 minute, 10 minutes, and 4 hours, respectively; d, dipping in alumina dust; e, rubbing with dust*

Days of incubation	Treatment	No. of eggs shrivelled after		
		30 min.	4 hrs.	24 hrs.
0–1	a	9	9	9
	b	10	..	..
	c	10	..	..
	d	2	2	10
	e	10	..	..
1–2	a	3	4	8
	b	7	10	..
	c	10	..	..
	d	0	0	8
	e	8	10	..
3–4	a	0	0	0
	b	8	10	..
	c	10	..	..
	d	0	1	3
	e	3	6	9
6–7	a	0	0	1
	b	7	7	8
	c	10	..	..
	d	0	0	2
	e	0	2	8

Permeability of the Shell Layer. Unwaterproofed eggs laid during the previous 24 hours were exposed to solutions of many different dyes, solvents, oils, and salts, but it was soon apparent that particles of a very wide variety of molecular sizes, and having either hydrophilic or lipophilic affinities, were capable of passing rapidly into the yolk. Stains, such as basic fuchsin, penetrate the shell layer and stain it intensely; and it is also stained if the dye is injected into the egg. Large protein molecules will also penetrate the shell. If, for example, the egg is immersed in laked blood for 24 hours the colour of the contents deepens as oxyhaemoglobin enters the yolk. Now it is known that the normal brownish tint of the *Ornithodoros* egg is due to the presence of haemoglobin pigments (Wigglesworth, 1943). As the shells of ovarian eggs at the stage of yolk deposition are no less permeable than one-day eggs, there is clearly no obstacle to the absorption of these pigments through the shell

layer from the haemolymph. The large pore size might also facilitate the penetration of the relatively small wax molecules into the lipophilic shell layer.

The waterproofed egg in the first day of incubation is, by contrast, entirely impermeable to all staining solutions unless they are incorporated with vigorous wax solvents. After treating the egg with the wax emulsifier Corg993 or cold chloroform for increasing periods, the permeability increases progressively and the staining properties of the shell layer are restored. At first the eggs swell osmotically in distilled water but fail to shrink in saturated sodium chloride, indicating that the shell is now permeable to small ions as well as to water. Extraction for 30 minutes in cold chloroform renders the shell permeable to sucrose but it still stains only in small and isolated patches. However, after extraction in boiling chloroform for 5 minutes the original permeability of the shell is completely restored and it then stains deeply and uniformly. These observations must imply that there is considerable affinity between the wax and the shell material.

Permeability of the Inner Membrane. Normal eggs incubated for 3 days at 25° C. (and therefore possessing a completed inner membrane) were extracted for 5 minutes in boiling chloroform. After this treatment the egg-shell as a whole was found to be permeable both to water and to monovalent ions, but, although the shell layer stained deeply with aqueous basic fuchsin, the dye no longer entered the yolk. This suggested that the inner membrane was permeable to water and salts but not to larger molecules.

The properties of the inner membrane may be tested more directly by carefully puncturing and stripping off the shell layer from the three-day waterproofed egg. The inner membrane, which is left entire as a delicate pellicle surrounding the yolk, is then found to be very permeable to water, as is shown by its swelling properties. Its outer surface, which is matt and seemingly homogeneous, is fairly readily wetted by water. On the other hand, if the shell layer is removed from an egg in the eighth day of incubation, the inner membrane is distinctly greasy and hydrofuge; and the egg swells much more slowly in distilled water.

Although this evidence does not entirely exclude the possibility that the inner membrane is impregnated from within by lipoid secreted by the embryo, it seems much more probable that the wax deposited on the outside of the egg gradually migrates through the shell layer as far as the inner membrane. Since the inner membrane is at first freely permeable to water, the diminishing influence of chloroform and abrasive dust on transpiration cannot be ascribed to the deposition of this layer. It is also noteworthy in this connexion that the effect of these agents gradually declines throughout the period of incubation—a result which might be expected from the slow infiltration of wax into the shell layer—whereas the inner membrane is laid down at a particular stage of incubation. These changes in the egg-shell during the incubation period are indicated diagrammatically in Text-fig. 12A, B.

If no secondary waterproofing layer is secreted after the onset of incubation, there should be no marked change in the critical temperature of the egg. The

vaporation curves of batches of eggs incubated for 6 days at 25° C. is shown in Text-fig. 4. The critical temperature is approximately 44° C. and compares with a value of 43° C. for the one-day eggs. Nevertheless, the lower rate of water loss above the critical temperature, which is brought out clearly in this figure, is a very constant feature of the older eggs. It is possible that we are observing here a further effect of the impregnation of the shell layer with wax (see p. 330).

DISCUSSION

Our observations have shown that the tick egg is waterproofed by an external covering of wax which is secreted by the female tick after the shell layers are complete. This is the reverse of the condition found in *Rhodnius* and other insect eggs where the impermeable wax layers are laid down by the oocyte or embryo after the formation of the chorion and therefore lie inside it (Beament, 1946b, 1948b). From the superficial position of the wax layer it follows that tick eggs are very readily attacked by wax solvents, detergents, or abrasive dusts; whereas these agents often exert a remarkably small effect on the transpiration from many insect eggs.

An additional property of the waxy covering is to cause the eggs to adhere in a cluster—a function often assumed by the cement-secreting glands in insects. Among the Argasidae the wax layer responsible for the impermeability of the general integument is protected by an external covering of cement (Lees, 1947). Nevertheless, the manner in which the egg is waterproofed is obviously incompatible with the acquisition of a further protective layer. And our results show clearly that the waxes are in fact freely exposed on the surface of the egg.

In *I. ricinus* wax is applied to the outside of the egg in two stages. An incomplete layer, secreted probably by the lobed accessory glands, is first smeared over the egg during its passage down the vagina; and a further complete covering of wax is applied when the egg touches Géné's organ. On the other hand, there is no evidence that the egg of *O. moubata* is even partially waterproofed before it is laid: here Géné's organ is solely responsible for secreting the waterproofing layer. This difference may possibly be related to the size of the egg. In *Ixodes* (as indeed in all Ixodidae) the egg is relatively small and has an approximate volume and surface area of only 0.045 mm.³ and 0.61 mm.² respectively, as compared with values of 0.33 mm.³ and 1.3 mm.² for the *Ornithodoros* egg (Text-fig. 13A, F). Whereas the rate of water loss from the egg may be expected to vary according to the surface area, the amount of water that can be lost before desiccation becomes critical, and also the rate of shrinkage of the egg, will be determined by the volume. It is indeed an observed fact that unwaterproofed eggs of *Ixodes* (but not those of *Ornithodoros*) shrink so rapidly that there would clearly be some danger of excessive desiccation taking place during the manipulations of the egg by Géné's organ, were the first incomplete wax layer not already present.

Evidence, both of an observational and experimental nature, shows clearly that the function of Géné's organ is to transfer a waterproofing wax to the

surface of the egg. The gland is a proliferation of the general epidermis which is folded inwards from the cuticle in this region. This relationship provides a simple explanation of the fact—which at first sight appears as a striking example of parallel physiological adaptation—that the eggs of a given species of tick have the same order of resistance to desiccation as the parent species itself. The production of a waterproofing agent is a general property of the epidermis and *Géné's organ* can be regarded as a region specialized for waterproofing the egg. Nevertheless, there are important differences in the properties of the egg and cuticular waxes which can, in turn, be related to their specific functions (see below).

A consideration of the nature of the wax precursors raises some interesting questions which are worthy of further study by more exact chemical methods. Although it has proved impossible to isolate the wax itself from this situation, it is highly probable that the wax precursor is present in the watery contents of the horns of *Géné's organ*. Protein is also present, but the nature of its association with the wax is not clear. Certainly the wax is not dispersed as a coarse oil-in-water emulsion stabilized by protein (in a manner analogous to the dispersion by protein of the poly-isoprene aggregates in rubber latex). Against such an interpretation may be cited the absence of visible droplets in the horn contents, the insensitivity to changes in pH and to the presence of excess polyvalent ions, and the stability to heat. An alternative suggestion that the horns may contain emulsifying agents, other than protein, receives no support from the experimental results, for the secretion from the horns is found to be entirely devoid of emulsifying properties when tested against the wax deposits from the outer surface of *Géné's organ*. It is more likely that the wax is intimately associated, and probably chemically linked, with protein when it is secreted by the gland. Although the nature of such lipo-protein complexes is but imperfectly understood (Chargaff, 1944), the action of proteins in dispersing water-insoluble lipids, like cholesterol, is well known.

The stability of the wax-protein association is such that it is difficult to believe that the wax could be liberated by any purely physical process, for example, by ultrafiltration through non-living cuticle. And the cuticular wall of the horns is devoid both of pore canals and of any cytoplasmic lining. We have shown, however, that pore canals, penetrating the cuticle, are present around the base of the horns where the gland epidermis becomes applied to the inner surface of the endocuticle; and there is also a zone where the pore canals remain uncovered by the cement which forms an external covering over the basal part of the stalk. If, as appears probable, this is the site of wax secretion on the outer surface of *Géné's organ*, the horns must serve as reservoirs for containing the relatively large quantities of wax precursor needed for the rapid waterproofing of a batch of eggs. Such a function must also imply that the precursor secreted by the gland is first stored in the horns and is then again taken up by the gland-cells as required and passed through the cuticle by the cells which maintain connexion with pore canals. The form in which the wax is transported through the cuticle is, of course, unknown,

out the highly soluble wax-protein complex would appear to be a material very suitable for transport by the cell. At the cell surface, perhaps at the tip of the pore canals, the complex appears to be broken down and the wax in some way released. The protein moiety must then be retained by the cell, for, as we have seen, the wax deposits on the outside of Gén 's organ are protein-free.

Since Gén 's organ makes contact with only a small part of the total surface area of the shell during normal oviposition movements, the newly secreted wax must have the ability to spread and complete the waterproof layer over the egg. We have shown that although wax from the deposits on Gén 's organ does possess definite powers of spreading on unwaterproofed eggs and, under very favourable circumstances, on prepared membranes, these powers are unexpectedly feeble. By comparison, for example, with the waterproofing grease from the cockroach cuticle (Beament, 1945), spreading of the egg waxes is slow and inefficient. The lipophilic properties of the shell material and the uniformly smooth surface of the shell layer are undoubtedly factors which favour spread. There is no reason to suppose, however, that the extension of the wax film is also assisted by the presence of specific spreading agents on the egg surface. Perhaps the wax undergoes further chemical changes after secretion, resulting in a partial loss of the spreading properties.

The comparison of the critical temperatures of tick cuticles and eggs also suggests points of interest in relation to the spread of waterproofing materials. In two species of *Ornithodoros* the critical temperatures of the egg, although comparatively high, are about 20  C. lower than are those for the cuticle of the parent species. These differences are correlated with the physical properties, the natural wax from the cuticle being hard and crystalline whereas that from the egg is soft and viscous. It would seem likely that the properties of the egg wax are largely dictated by the need for spreading over the surface of the shell. In general, it has been found that species of insects with high critical temperatures are waterproofed by hard apolar waxes (Wigglesworth, 1945; Beament, 1945). One might suggest from the knowledge at present available that a wax with good spreading powers is unlikely to have a high critical temperature; and that this property will be achieved only at the expense of some increase in permeability. Unlike the egg waxes, the cuticular lipoids have little need for mobility as spreading may be limited to the distance between neighbouring pore canals. Among Ixodidae there are comparatively slight differences only between critical temperatures of cuticle and egg in the same species. Nevertheless, preliminary observations suggest that the waxes concerned differ in their physical properties as widely as in the Argasidae; the egg waxes always appear to be more mobile.

A notable feature of the development of the tick egg is the fact that the shell is secreted not by follicle cells, but by the oocyte itself. The microscopic structure of the shell is comparatively simple. Only three layers are present when the egg is laid, the outer wax layer, a layer of granules with reducing properties, and the shell layer; a fourth layer, the inner membrane, which,

like the shell material, is composed of protein, is added after several days of incubation.

The unwaterproofed shell layer is remarkably permeable both to water and to large molecules with either lipophilic or hydrophilic affinities. Our experimental results point to the conclusion that the mobile wax gradually invades and impregnates the shell layer and in places reaches the inner membrane. The changes in the permeability of the egg around the critical temperature are probably related to these events. It has been suggested that increased transpiration through the insect cuticle at the critical temperature is associated with the destruction by thermal agitation of the parallel alignment of the wax crystallites at the protein-wax interface (Beament, 1945). If the shell layer is pictured as a loose protein meshwork, the wax will at first be entirely superficial and temperature will exert a correspondingly profound effect on transpiration. As the wax infiltrates into the shell layer and not only occupies the protein interfaces throughout the thickness of the layer, but also fills the intermolecular pores, the permeability to water may be expected to fall. This may be the explanation of the lower transpiration rate of 6-day eggs above the critical temperature. There is no evidence that a similar relationship is ever established between the cuticular waxes of ticks and the cuticulin-polyphenol substratum on which they are deposited. Here the waterproofing layer remains entirely superficial and therefore continues to be accessible to such agents as abrasive dusts (Lees, 1947).

SUMMARY

1. During the oviposition of ticks a glandular organ—the organ of Gén  —is everted and touches the egg. If it is prevented from everting most of the eggs shrivel rapidly; few hatch even in a humid atmosphere.
2. The waterproofing properties of the normal egg are conferred by a superficial coating of wax, $0.5\text{--}2.0\ \mu$ in thickness. In *Ornithodoros moubata* the wax is secreted and applied solely by Gén  's organ. In *Ixodes ricinus* waterproofing takes place in two stages: an incomplete covering of wax, probably secreted by the lobed accessory glands, is first smeared over the egg during its passage down the vagina; waterproofing is then completed by a further application of wax from Gén  's organ after the egg has been laid. Owing to its superficial position on the egg the wax layer is readily attacked by solvents and emulsifiers.
3. The morphology of Gén  's organ in *O. moubata* is described. The gland is a proliferation of the epidermis which lies detached from the cuticle. Its secretion, a watery refractile liquid containing the wax precursor, accumulates between the gland and the cuticle in two horn-like extensions. The wax is probably secreted through pore canals distributed over a narrow zone of cuticle below the horns; the cement covering-layer of the epicuticle does not extend to this zone.
4. The transparent, heat-stable material isolated from the horns of Gén  's organ is regarded as the wax precursor. Solubility in water is probably con-

ferred by chemical linkage with protein. The precursor is taken up from the horns, where it is stored, and is presumably broken down within the gland cells. The wax is then secreted through the pore canals while the protein moiety is retained by the cell.

5. The critical temperatures of the eggs of Ixodidae range from 35° C. in *I. ricinus* to 44° C. in *Hyalomma savignyi*; only slightly higher critical temperatures were recorded for Argasidae (45° C. in *O. moubata*). Eggs with lower critical temperatures are more susceptible to desiccation. The susceptibility of the eggs of a given species is of the same order as that of the parent species; but whereas in Ixodidae the critical temperatures of the egg and the cuticle of the female tick are approximately the same, in Argasidae the critical temperatures of the cuticle are much higher (62° C. in *O. moubata*). These differences are related to the physical properties of the waxes. The cuticular wax in *O. moubata* is hard and crystalline (m.p. 65° C.), whereas the egg wax is soft and viscous (m.p. 50–54° C.).

6. The natural wax from Géné's organ has definite powers of spreading on the surface of the egg and so completing the waterproofing layer.

7. The material extracted with boiling chloroform from egg-shells or from nymphal cuticles separates spontaneously into two fractions, a hard white wax (c. 85 per cent. by weight) and a soft yellow grease (c. 15 per cent.). The properties of these two lipoids differ conspicuously from those of the natural wax. Attempts to deposit the extracted materials on membranes in the form of a waterproofing layer were unsuccessful.

8. Ovulation is described in *O. moubata*. The shell of the tick egg is secreted by the oocyte itself and not by follicle cells. Three layers can be distinguished in the 24-hour egg: (i) an outer wax layer; (ii) an incomplete layer of granules which reduce ammoniacal silver nitrate; (iii) a shell layer. A fourth layer, the inner membrane (iv), is secreted by the oocyte after incubation for 2–3 days.

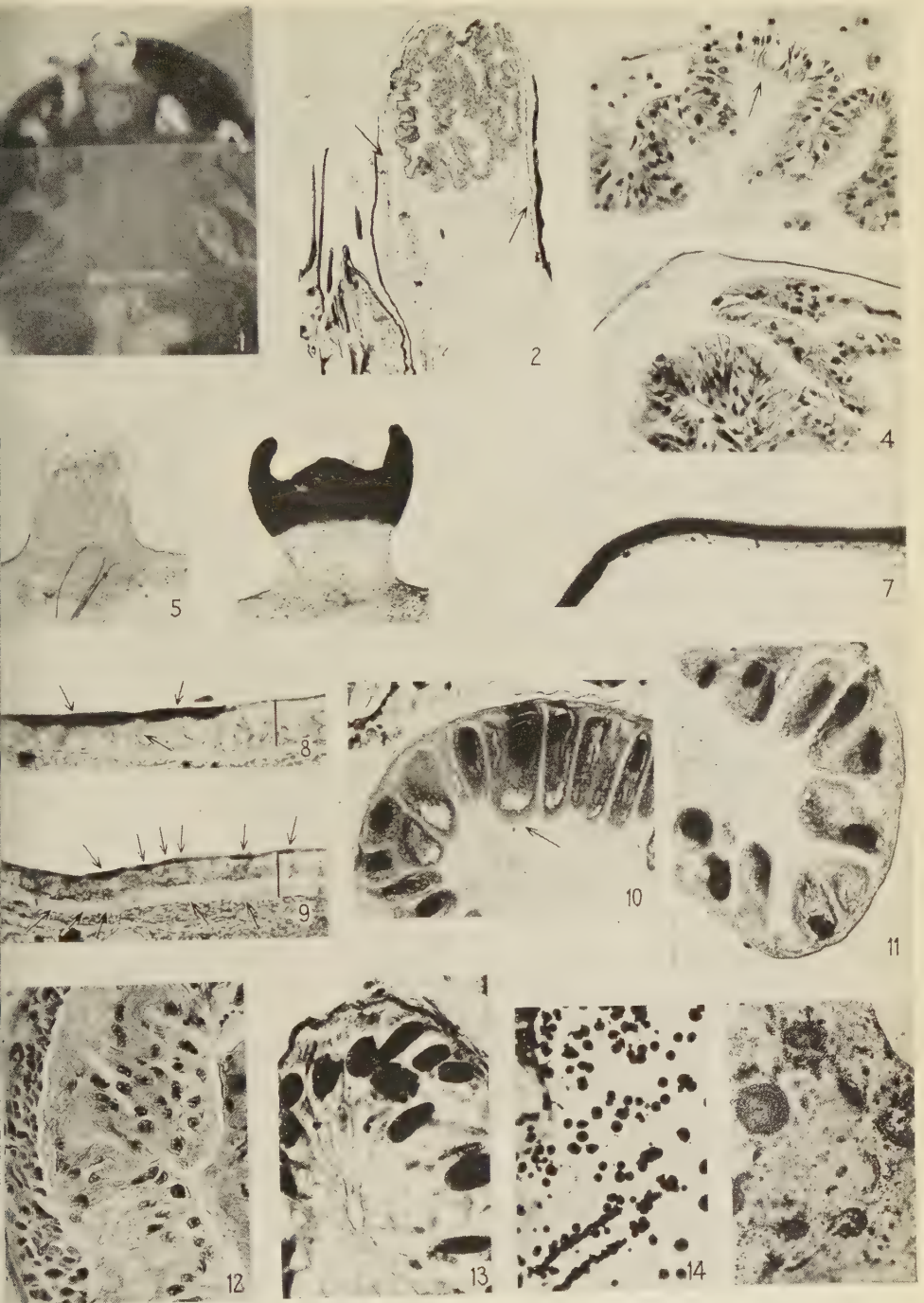
9. Both the shell layer and the inner membrane are composed of resistant, elastic protein and are devoid of chitin. The shell layer of the unwaterproofed egg is highly permeable to water and to large molecules with either hydrophilic or lipophilic affinities. The inner membrane is at first freely permeable to water and to inorganic ions. During the course of incubation the wax gradually migrates into the shell material and may reach the inner membrane. As this occurs, the effectiveness of abrasive dusts and of chloroform in promoting increased transpiration through the shell is notably reduced.

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EXPLANATION OF PLATE I

- Fig. 1. *O. moubata*. Female tick, with Géné's organ everted, clipped between glass slides. $\times 7$.
 Fig. 2. *O. moubata*. Sagittal section through the centre of an everted Géné's organ to show continuity of epidermis (indicated by arrows) with the gland. $\times 50$.
 Fig. 3. Géné's organ in *O. moubata*. Gland epithelium showing secretory droplet. $\times 210$.
 Fig. 4. The same, showing relation of gland to the thin cuticle of the horns. $\times 210$.
 Fig. 5. Whole mount of Géné's organ in *O. moubata*. The tick with the organ everted was immersed for 30 minutes in ammoniacal silver nitrate. Note absence of staining. $\times 20$.
 Fig. 6. A similar organ extracted for 30 minutes in cold chloroform and stained with ammoniacal silver. Note intense staining over the horns where cement is absent. $\times 20$.
 Fig. 7. *O. moubata*. Cuticle of the horns heavily stained with silver after chloroform extraction. There are no pore canals in the thin unstained endocuticle. Section photographed in water. $\times 430$.
 Figs. 8, 9. *O. moubata*. Sections of the cuticle from the zone between silver-staining and non-silver-staining areas of Géné's organ. The thickness of the cuticle is indicated by an ink line, pore canals in the endocuticle and silver-staining areas of the epicuticle by arrows. $\times 430$.
 Fig. 10. *I. ricinus*. Section through one lobe of the gland of Géné's organ. Honeycomb border indicated by arrow. Iron haematoxylin. $\times 210$.
 Fig. 11. *I. ricinus*. Portion of lobed accessory gland, to show similarity with gland epithelium of Géné's organ. Iron haematoxylin. $\times 210$.
 Fig. 12. *I. ricinus*. Longitudinal section of vaginal cells and circular muscles. Iron haematoxylin. $\times 210$.
 Fig. 13. Transverse section of tubular accessory gland. Iron haematoxylin. $\times 210$.
 Figs. 14, 15. *O. moubata*. Surface view of shell fragments from two unwaterproofed eggs treated with 5 per cent. ammoniacal silver nitrate. $\times 210$.



A. D. LEES AND J. W. L. BEAMENT—PLATE I

A Study of the Spheroid System of Sympathetic Neurones with Special Reference to the Problem of Neurosecretion

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With two Plates

INTRODUCTION

IN the year 1891 Camillo Golgi of Pavia in a short article entitled 'Sur la structure des cellules nerveuses' announced the discovery of his now-famous 'Apparato Reticulo Interno'. He states:

L'aspect caractéristique de cet appareil réticulaire interne peut provenir de la forme prédominante en ruban, des fils, du mode de se diviser, de s'anastomoser et du cours de ceux-ci (spécialement dans les grandes cellules on observe un cours nettement tortueux), de la présence dans cet appareil de minces plaquettes ou de petits disques arrondis et transparents dans le centre, qui forment comme des joints nœuds du réseau, et enfin de la couleur spéciale, jaunâtre, que prennent les fils par effet de la réaction.'

This celebrated discovery as so often happens was made by chance, the new 'apparatus' appearing at the behest of the 'black reaction' which occurs when nervous tissues previously soaked in a solution of dichromate are placed in silver nitrate.

Since the time of Golgi the homologues of this nerve-cell 'apparatus' have been studied in many kinds of cells by a great number of workers (Hirsch, 1939, in his recent monograph lists over 2,000 references to this topic).

Because of the great diversity of form the apparatus may take when studied by the classical techniques of silver and osmium impregnation in diverse cells, or even in identical cells within a tissue, authors frequently fail to reach agreement on the precise identification of the apparatus. Confusion has been the inevitable result and in some quarters a degree of scepticism prevails even to this day concerning the existence of this organella—the sceptics ascribing the appearance in the histological preparations to artifacts of technique; a view strengthened by the difficulty of adequately studying the apparatus in the living cell.

In an attempt to resolve this morass of conflicting opinion, Baker (1944) set out to find whether the fully developed Golgi element of diverse cells could be shown to conform to a single structural plan. His researches led him to conclude that in the nerve-cell this element consists not of elaborate peri-

nuclear networks but of separate discrete or 'dispersed systems' lying in the cytoplasm. These systems consist of a lipoid sheath or pellicle enclosing a more or less fluid core which can be stained during the life of the cell with neutral red chloride. Further, he indicated that this binary system of bodies may occur widely in other tissues and there represent the true Golgi complement of the cells.

Baker preparations of nerve-cells are indeed objects of great interest and have led to considerable speculation to the neuro-histologist. The appearances are novel and cannot easily be reconciled with the classical Golgi networks of the Golgi apparatus, so much so that some workers would rather incline to the view that a completely new cytoplasmic organella has been demonstrated in the nerve-cell.

Consequently it has been thought advisable to carry out a further investigation of this problem and to try to establish the identity of the Baker bodies with the one hand with structures visible within the living cell, while on the other with the classical appearances of the silver and osmium techniques. The interesting new technique of phase-contrast microscopy has been utilized as far as possible in this investigation.

METHODS

The freshly excised superior mesenteric ganglia of mice were examined in a few drops of 0.7 per cent. sodium chloride containing 0.2 per cent. of 10 per cent. anhydrous calcium chloride; the fluid was warmed to 37° C. in a incubator. Both transmitted and phase-contrast illumination were employed in this study. The details of the phase-contrast microscope used will be described in a forthcoming article by Kempson, Thomas, and Baker.

Both supravital and intravital staining were carried out with neutral red chloride B.D.H., methylene blue B.D.H., and Janus black B.D.H. dissolved in the required concentrations in 0.7 per cent. sodium chloride solution containing 0.2 per cent. of 10 per cent. calcium chloride. For intravital staining 2 c.c. of a 1 per cent. solution of neutral red chloride in distilled water was injected intraperitoneally following a preliminary ether induction. Coal-gas was used for killing the animals.

For fixed material the following techniques were employed:

1. Baker (1944) sudan black technique for the demonstration of the Golgi apparatus in frozen sections.
2. The variant of the above technique applicable to paraffin sections as described on p. 340.
3. Mann-Kopsch technique.
4. The Azan stain of Heidenhain and Masson's trichrome stain following Zenker Formol fixation and post-chroming at 37° C. for 3 days in a saturated solution of potassium dichromate in distilled water.
5. Feulgen reaction.
6. Celestin blue-pyronin reaction of Sanders (1946).

The Living Cell

OBSERVATIONS

The superior mesenteric ganglion of the mouse was found to be a very suitable tissue for high-power microscopic observations of the living sympathetic neurone. The ganglion was rapidly dissected with the aid of a Greenough binocular microscope and placed in a capsule of warmed 0.7 per cent. sodium chloride containing 0.2 per cent. of 10 per cent. anhydrous calcium chloride. Because of the small size of this ganglion and its general transparency further teasing of the material is unnecessary. If the unsupported coverglass is placed upon the tissue the resulting pressure is sufficient adequately to flatten the cells and thus allow a clear microscopic image of the cytological details of the surface cells to be obtained.

The cytoplasm of the ganglion cell is seen to contain a variable number of small roundish bodies more or less evenly distributed throughout. These bodies vary in size from single small spherical homogeneous granules to larger compound objects which appear to be made of two parts, a somewhat yellowish spherical core to which are attached around the periphery smaller darker grains or crescentic caps. The largest bodies are frequently somewhat irregular in contour and are closely similar to, although smaller than, the 'mulberry spheroids' described in *Helix* neurones (Thomas, 1948). In this respect the general similarity of structure of the living vertebrate sympathetic neurone to the living invertebrate cerebral neurone is indeed very striking. Further, if one only examined preparations fixed in Formol-saline or Bouin's fluid stained with haematoxylin and eosin the impression would be gained that these cells possess a clear homogeneous cytoplasm whereas in point of fact the exact opposite is true: the living cell is crowded with a large assortment of diverse granular bodies scattered throughout its cytoplasm.

Vital staining with Neutral Red and Janus Black

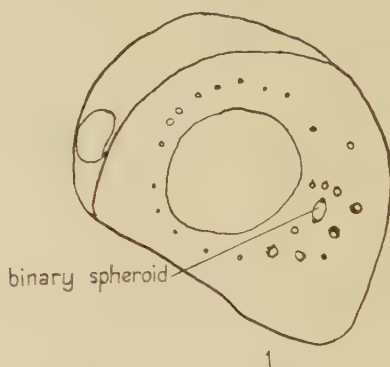
Neutral red chloride 1-10,000 in saline is a very valuable dye for clearly revealing these intracellular bodies. In a short while after its application to the tissue the spherical bodies absorb the dye and become tinged a red colour throughout.

It must be stressed that the neutral red solution merely colours pre-existing bodies which can be readily seen within the unstained cell. There is no suggestion, under the conditions of the staining experiment outlined in the 'Methods' section of this paper, of the formation of unspecific neutral red segregation droplets within the cells. Exactly the same is true for *Helix* neurones.

As the colour begins to fade from the vitally stained tissue owing to the reduction of the dye to its leucobase the duplex bodies are particularly well differentiated into their component parts—a bright staining pellicle to a more or less faintly tinged core. Apparently the pellicle, which in some cases appears as a complete skin to the core and in others as attached granules or

crescentic caps, has a far greater capacity once stained to hold the dye and to resist its eventual reduction.

Methylene blue was similarly applied both vitally and *intra vitam* as this dye has been advocated by Worley (1943) as a stain of high specificity for the 'externum' of the Golgi systems of a number of cells studied by him and his co-workers. In my hands his methods gave results which were exactly comparable to those given by neutral red. In some cases the differentiation of the duplex bodies is even more striking. Text-fig. 1 is an accurate camera-lucida drawing of a cell stained with methylene blue. It will be noticed that the larger systems may measure 2μ – 3μ in diameter. The smallest methylen-



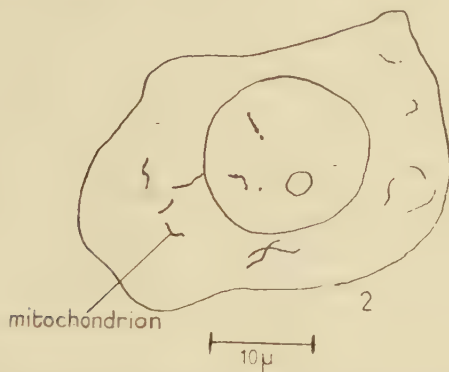
TEXT-FIG. 1. A mouse sympathetic neurone stained vitally with methylene blue. The spheroids exhibit attached blue-staining granules and crescents. Camera-lucida drawing.

blue-stained granules are apparently homogeneous or at least are too small for accurate description as binary systems. Similar bodies have been seen by Hirsch (1940) in *Ascaris* and interpreted by him as remnants of the Golgi apparatus (Golgi 'Rest') which serve as a reserve of material apparently capable of development into binary systems as circumstances require.

Janus black possesses the property of staining very specifically the mitochondria to the exclusion of the spheroidal bodies. This technique although difficult will often give good results if repeated trials are made. The mouse ganglia were again chosen for their small size and transparency which allow observation of intact cells undisturbed in relation to their surroundings. Suitably stained cells reveal the presence of numerous delicate filaments or moniliform chains tinged a greenish-black colour. In the most successful preparations the mitochondria curiously appear to be situated immediately beneath the cell membrane, an appearance probably attributable to the fact that these cells have been but slightly penetrated by the dye and only the peripherally situated mitochondria are stained and clearly differentiated against an as yet clear central mass of cytoplasm containing the remaining unstained mitochondria. In the same way stained cells fail to reveal more deeply situated mitochondria as the very delicate filaments do not concentrate

enough dye to 'show through' a thick layer of slightly diffusely stained cytoplasm. Text-fig. 2 is a camera-lucida drawing of a mouse ganglion cell stained vitally with Janus black and showing numerous mitochondrial filaments. This drawing should be compared with Pl. I, fig. 4, which shows exactly similar objects in the fixed cell after post-osmification in the Mann-Kopsch technique.

Janus green B, one of the most popular dyes for showing mitochondria in living cells, was found unsuitable for mouse cells. As always with Janus



TEXT-FIG. 2. A mouse sympathetic neurone stained vitally with Janus black. The filamentous mitochondria are stained a greenish black. Camera-lucida drawing.

staining, dyes of different origins and at varying concentrations should always be applied in order to obtain the most satisfactory results.

Fixed and Stained Material

Reactions to osmium tetroxide. If small pieces of mouse ganglia are suspended in saline as a hanging drop on a cavity slide, and a small drop of 2 per cent. osmium tetroxide solution placed in the cavity but arranged so as not to touch the hanging drop (Text-fig. 3), the effects of osmic vapour can be studied on the spheroid bodies while fixation of the living cell is taking place. After a period of a few hours these bodies assume a brownish colour.

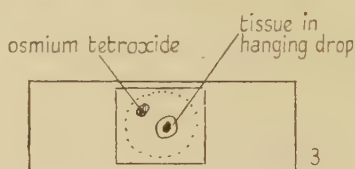
If tissues are immersed in a simple solution of osmium tetroxide and left overnight in the incubator the spheroids can be totally blackened.

In order to study what part if any these osmiophil bodies may play in the subsequent changes known to occur in the cell during prolonged osmification, it was decided to carry a number of pieces of tissue through one of the classical osmium techniques for the demonstration of the Golgi apparatus, and to withdraw and examine some of the pieces at regular intervals during the stated period (usually 6–10 days) of impregnation. The Mann-Kopsch technique was chosen for this experiment, pieces of ganglion being fixed for 2 hours in Mann's mercuric-chloride-osmium solution and transferred after

washing to 2 per cent. osmium tetroxide and placed in the 37° C. incubator. At 12-hourly periods a piece was removed, washed, dehydrated, embedded and cut at 7 μ .

The discrete spheroids are plainly visible in the first batch of sections after a total of 12 hours' immersion. Very frequently in suitable cells one can find the appearance shown in Pl. I, fig. 3. The smallest granules are homogeneously blackened, but in the case of the larger ones only their outer coats are impregnated, revealing a more or less colourless centre. Frequently this osmiophilic pellicle shows local thickening which appears as caps or crescents applied to the spheroids. Sometimes linear bands occur around the core.

After 48 hours, nearly all the spheroids are totally blackened, only the largest bodies retaining the clear central area. As the degree of osmification



TEXT-FIG 3.

of the cells varies considerably within any one section, material left for 3 days will usually show all these stages from incomplete to complete blackening of the spheroids in the same section.

Within 24 hours in addition to the spheroid bodies a second osmiophilic element in the form of delicate filamentous threads becomes visible in the cells (Pl. I, fig. 4). At first these threads are but faintly tinged a light greyish colour, but as osmification proceeds their colour darkens and the objects appear thicker, longer, and more tortuous. Adjacent filaments soon appear to coalesce to form long wavy bands which frequently appear to fuse with the already blackened spheroid bodies which then become incorporated as so many nodular swellings upon the bands (Pl. I, fig. 5). Lateral anastomoses of these nodular bands occur as more and more reduced osmium is superimposed in a non-specific manner upon this scaffolding originally formed by two separate discrete elements, the filamentous threads and spheroid bodies. In this way an apparently unrelated and new structure is built up within the cell which at first sight bears no relationship whatsoever to any objects visible within the living cell. The life-like appearance of the cell seen after short periods of osmification (12–36 hours) is altogether lost as the Mann-Kopsch process proceeds with its snowball-like deposition of osmium throughout the cell. That there is a general non-specific deposition of osmium particles throughout the cell seems certain from the generalized gradual darkening of the cytoplasm as a whole as osmification proceeds. Eventually the whole cell can be blackened throughout and made quite opaque if tissues are left for a long time (10–20 days) in osmium solution at raised temperature.

(37° C.). It seems reasonable to assume that much of this non-specific deposition of osmium particles would take place first about a centre of already impregnated intracellular objects, and that this centre would itself be transformed in a haphazard manner into a thickened, distorted, and unrecognizable object as superimposed layers of metallic particles are built up upon it.

After 6 days the great majority of the cells exhibit the classical Golgi appearance (Pl. I, fig. 6). The cell shows a generalized blackening but more especially around the anastomotic network of impregnated strands. In some cases the originally single spheroid bodies are completely lost within the 'black reaction'. In other cells they can still be discerned either incorporated within the meshwork itself or lying between the strands.

The Application of Sudan Black to the Fixed Cell

Baker (1944) introduced a new method for the study of the Golgi element of cells. He applied the substance sudan black, which strongly colours the lipoidal external pellicle of the system so as to differentiate these bodies in frozen sections. Rabbit anterior mesenteric ganglia were chosen for study as they are considerably larger than in the mouse and their frozen sections are large enough to afford easy handling with the technique. The formal calcium sudan method applied to these cells reveals the presence of a number of small clear vacuoles scattered throughout the cytoplasm. Each vacuole is bounded by a delicate sudanophil skin which is characteristically concentrated and heaped up on one side, giving to the whole body a signet-ring appearance. Sympathetic neurones prepared by this method have a very novel appearance which cannot at first sight be reconciled with any of the classical cytological pictures of the neurone. One may almost be excused in interpreting the vacuole with its lipoid skin as a new nerve-cell organella.

The vacuoles vary fairly considerably in size within any one cell and correspond in size and general distribution with the spherical bodies observable within the living cell and stainable vitally with neutral red. The neutral red staining caps or crescents in the pellicle of the living duplex system very closely resemble the appearances of the 'heaped-up' sudanophil rims to the vacuoles.

As in the case with the smallest osmiophil bodies a clear vacuole cannot always be resolved in the centre of the body. Such objects appear as very small sudanophil grains just resolvable with the microscope and would correspond to the 'Praesubstanz' or 'Rest' of Hirsch.

It must be stressed, however, that the formal-calcium sudan black technique as applied to frozen sections fails to reveal within the sympathetic cell any obvious deposits of lipoidal granules, as may frequently be found in resting gland cells or such as the lipochrome deposits of invertebrate neurones which are always strongly sudanophil.

Deposits of black 'melanin' granules are sometimes described in sympathetic cells. I have never found them in rabbit ganglia.

Attempts were made to develop a technique to preserve these sudanophilic vacuoles in paraffin sections. The following method proved a useful one for this purpose:

1. Fixation in acetic-osmic-bichromate of Bensley's or Helly's fluid followed by post-chroming at 37° C. with a saturated aqueous solution of potassium dichromate. (The dichromate salts conserve the lipines by converting them into alcohol-insoluble substances which remain sudanophilic after embedding.)
2. Wash 4–6 hours at the tap.
3. Dehydrate and embed in paraffin. Cut and mount sections on slides with albumen solution in the usual manner.
4. Take the section down through xylene and the alcohols, to 70 per cent alcohol—leave 3 minutes.
5. Place in saturated sudan black in 70 per cent. alcohol 7–10 minutes.
6. Quickly rinse in 50 per cent. alcohol to remove surface stain particles.
7. Rinse in water, counterstain in carmalum, and mount in Farrant's medium.

With this technique the cores of the spherical bodies appear as faint yellowish-brown masses and not clear vacuoles as in the frozen sections. Closely applied to the core are a number of strongly sudanophil granules or crescents. Sometimes there is a more or less complete sudanophil pellicle to the core but the more general appearance is of attached granules, whereas in the frozen sections crescents predominate. The largest spheroid bodies often have a large number of dense black granules almost completely covering their surfaces.

Careful focusing is necessary in order to see the yellowish-brown cores within the cluster of granules.

Pl. I, figs. 1 and 2, are a photomicrograph and a drawing of a cell exhibiting a number of spheroids with their black sudanophil pellicle. The core of the two largest spheroids can be distinctly seen surrounded by numerous small granules. Such large mulberry forms occur frequently and appear to have a somewhat irregular outline and uneven contour suggestive of their being possible aggregates of smaller spheroids.

Some cells show large numbers of small sudanophil granules often aggregated into clumps at one pole of the cell. Usually a few typical spheroid systems can be seen within this clump. It may be argued that some of these small granules could be Rests from which new systems might be formed, but as a similar appearance does not occur in control material after formal-calcium fixation it seems more likely that they are precipitates of cytoplasmic material invisible in the living material. Since these sudanophil granules only occur following the use of fixatives containing protein precipitants, the assumption is that they must constitute in life a lipoprotein complex which is split by the fixative into its constituent parts, the fatty element being thereby unmasked and preserved in the sections by the action of the dichromate. Pl. II, fig. 1.

illustrates a clump of these granules following Helly's fixation and stained with sudan black.

Reactions of the Fixed Cells to the Trichrome Techniques

In view of the interesting researches of Scharrer and others upon the intraneuronal 'secretion' granules, the Azan and Masson trichrome techniques were applied to paraffin sections of Helly-fixed rabbit sympathetic ganglia.

Morphologically two types of stainable granules can be seen in the cells by the application of these techniques, viz.:

(1) Perfectly spherical discrete globules showing variations in size from very small to medium-sized granules staining red with either azocarmine or the acid fuchsine of the Masson technique.

(2) Much larger irregular granules having a mulberry or compound appearance. Sometimes a large granule may be surrounded by an almost complete ring of much smaller granules in very close contact with it. Sometimes as many as four or five large mulberry granules may occur together within one cell (Pl. II, fig. 3). These bodies similarly stain with azocarmine or acid fuchsine.

(3) Clumps of ill-defined brownish-yellow granules occur in some cells. They are usually gathered together characteristically in one pole of the cell (Pl. II, fig. 6). These granules only occur following fixation with protein precipitants when combined with dichromate and are of a fatty nature as they stain with sudan black. They are discussed above and will not be referred to in this section. They do not appear in tissues fixed in lipid solvent fixatives such as Carnoy's solution.

The azocarminophil and fuchsinophil granules are of regular occurrence throughout the tissue. These granules can also be stained well with Heidenhain's iron haematoxylin. They give a negative reaction to Sakaguchi's arginine reaction but a mild positive xanthoproteic. They are negative to both the Feulgen and celestin blue pyronin reaction (see Sanders, 1946).

With the latter technique the Nissl substance is particularly well shown and does not bear any positional relationship to the granules described above. One of the most striking and curious features concerning these granules in the fixed cells and a very frequently occurring phenomenon is illustrated in Pl. II, figs. 4, 5, 6. Here an azocarmine-staining granule appears stuck upon the nuclear membrane, which itself is strikingly modified for a considerable distance on each side of the zone of contact. With the Azan technique this thickened zone of the nuclear membrane is usually coloured purplish-blue in contrast to the brilliant red granule. The photographs give but a poor impression in monotone of the striking appearance.

In some instances the granule appears to be partly within the nucleus itself, suggesting to the observer that the granule was either passing in or out from cytoplasm to nucleus or vice versa when the cell was fixed (Pl. II, fig. 6; the granule referred to is the black object shaped like a thick plus sign, lying on the nuclear membrane).

Occasionally similarly staining but smaller granules can be seen lying within the nucleus itself, where they can be differentiated from the single large nucleolus by their smaller size and tinctorial properties.

The nucleolus not infrequently loses its somewhat woolly spherical form and is modified into a somewhat fan-shaped structure which approaches and becomes contiguous with the nuclear membrane from within (Pl. II, fig. 7). This phenomenon is not associated with any modifications of the nuclear membrane as is the case when a cytoplasmic granule is applied to it.

It was felt desirable to correlate if possible the granules seen in the fixed and stained cell with the spheroid bodies and granules visible in the living cell and stainable in a differential manner by the vital dyes, neutral red and methylene blue, and by the sudan black method on frozen sections.

The technique described above, whereby sudan black is applied to paraffin sections followed by mounting in Farrant's medium, serves as a valuable means of bringing about this correlation. The following steps were carried out:

1. Fix material in Helly's fluid and post-chrome for 3 days at 37° C. in a saturated solution of potassium dichromate. Section by the paraffin method and colour with sudan black. Mount in Farrant's medium.
2. Photograph suitable cells and record their position with the stage vernier (Pl. II, fig. 1).
3. Remove the coverglass and leave in running water to remove the Farrant's medium (5 minutes).
4. Extract the sudan black by placing the slide in 70 per cent. alcohol for 5 minutes.
5. Place in water 1 minute.
6. Stain the section according to the Azan technique. Mount in balsam and re-photograph and compare the same cells (Pl. II, fig. 2).

In this way it can be shown that the azocarmine-staining granules possess a sudanophil sheath or coat which can at once be identified with that of the spheroid binary systems present in the living or vitally stained cell and with the vacuoles with lipid skins demonstrated in frozen sections.

In other words, this staining experiment proves that the granules, which are so strikingly shown with the trichrome stains, form within the core to the spheroid systems possessing lipoidal sheaths, as described previously.

DISCUSSION

The True Form of the Golgi Apparatus

As a natural development from the pioneering researches of Parat (1921) within recent years, much of the confusion has been resolved concerning the cellular organella usually described as the Golgi apparatus. From the important researches of Hirsch (1939), Worley (1943, 1944), and Baker (1944) many new facts have been brought to light on this vexatious problem, and it

becoming increasingly clear that this important zone of the cell consists of a number of discrete binary systems consisting of a lipoidal pellicle enclosing a more or less fluid core. This core constitutes the 'vacuome' of Parat and it is thought forms the site of production of most endocellular secretion antecedents, i.e. Golgi products.

The methods of attack on this problem have altered materially within recent years, and a new school of cytologists has developed which no longer bases conclusions on the structure of the Golgi system solely from the observation of its appearance in fixed material impregnated by the older classical silver and osmium methods.

The phase-contrast microscope has provided biologists with a tool particularly applicable to the study of the living cell. In the author's hands this instrument fails to reveal any structure within living nerve-cells, either invertebrate or vertebrate, that can be reconciled with the classical dictyosomes (Gatenby) or Golgi networks of the metallic impregnation technique, and he feels that another blow, perhaps a fatal one, has been dealt to earlier doctrines of workers such as Beams (1931), Hirschler (1918), Gatenby (1924), and others who have in a wide variety of studies upheld the classical teachings of Golgi (1891), Veratti (1898), and Cajal (1915).

In this study living vertebrate sympathetic neurones (of mouse and rabbit) have been shown to contain a number of discrete bodies which range in size from small grains through simple spheroids to larger bodies possessing an irregular contour.

The following facts have been established about these bodies:

1. They are easily visible in the living cell, appearing slightly yellowish with transmitted light.
2. They are stainable vitally with neutral red or methylene blue. As the colour fades from the preparations the bodies are differentiated into two parts, a pellicle and core.
3. They are clearly differentiated by Janus black or Janus green B in the living cell from the filaments or moniliform chains of the mitochondria.
4. They are tinged a brown colour with osmic vapour or totally blackened if immersed in a solution of osmium tetroxide.
5. They appear as vacuoles with lipoidal skins showing local thickening in frozen sections fixed in formal-calcium solution and when coloured with sudan black.

In the past it has been generally considered that valuable information on the Golgi could only be gleaned from tissues that had remained in osmium or silver solutions for a 'specified' time (with the osmium methods usually 6-10 days). At the conclusion of the procedure, should the tissues fail to reveal the established appearances it was usually assumed that the failure was due to 'over-' or 'under-' impregnation. In short, the older Golgi technique could be described largely as a cytological ritual to which the would-be-

successful technician must conform. It is surprising that more attempts have not been made to study the changes within the cell from the living state to the 'completed impregnation'.

It has long been acknowledged that the osmium Golgi techniques are superior in reliability to the silver methods. Consequently in this research the Mann-Kopsch technique was chosen for such an analytical study.

The experiments with this technique show very clearly that the most life-like picture of the cell is produced by very short periods of osmification, namely, 12–18 hours. Then the binary nature of the spheres is clearly differentiated as the pellicle is much more osmiophil than the core, but as osmification proceeds this appearance is lost and they become homogeneously blackened.

The filamentous mitochondria are similarly osmiophil and when they first appear in the Mann-Kopsch technique their size and configuration closely match their appearance in the living cell stained with Janus black. Apparently the mitochondria form the material basis for the subsequent progressive deposition of further particles of reduced osmium, for as the osmification proceeds the individual filaments are thickened and lengthened until finally adjacent threads appear to anastomose and link up one with the other in an irregular fashion.

The spheroid bodies in the main lose their general characteristics and become a dispersed system of individual bodies and become either incorporated in the tangle of thickened threads or are lost in the concomitant non-specific deposition of osmium particles which has taken place throughout the cytoplasm but more especially about the original mitochondrial framework. In this way at the end of 6–10 days these preparations reveal a picture at once identifiable with the classical Golgi network.

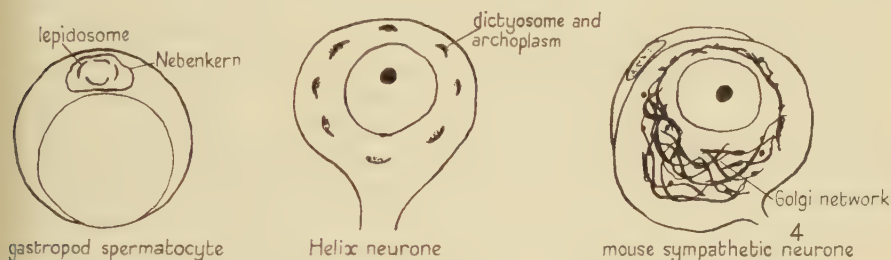
These results conform to the view that the classical Golgi network of the sympathetic neurone is an appearance produced in the cell in an artificial manner, namely, by a non-specific deposition of metallic particles about and upon two distinct categories of pre-existing intracellular organelles, the spheroid systems and the mitochondria.

I associate my results with the views of Hirsch, Worley, and Baker, from their studies of a number of diverse cells, and conclude that in the sympathetic neurones the Golgi substance consists of a number of dispersed binary systems possessing lipoidal chromophil capsules to a neutral red staining core—the vacuole of Parat.

It is indeed fascinating to read the original description of the apparatus from Golgi's pen in his article of 1898 (see introduction to this paper). It is very curious that his 'little rounded discs' which he describes as interposed upon the network like nodes, and 'transparent in the centre', should have for so long escaped the attention of the scores of subsequent investigators, whose attention seems to have been mainly directed to conserving the 'network' in their preparations to the detriment of the 'discs with transparent centres'—a component readily accessible in the living cell.

The Homology of the Golgi Element in Invertebrate and Vertebrate Neurones with the Golgi Elements of the Germinal Cells

The rod-shaped lepidosomes of the reproductive cells of Gastropods have long been claimed as the homologue of the Golgi apparatus of the neurone (Hirschler, 1918; Gatenby, 1924; and others). To support this claim the dictyosomes and archoplasm of the invertebrate neurone have been put forward as an intermediate link or transitional form of the apparatus. According to this view the individual rodlets of the dictyosome correspond to dispersed pieces of the vertebrate Golgi network, whereas the attached archoplasm represents a remnant of the condensed cytoplasm of the invertebrate Nebenkern. These views can be briefly summarized by the following schematic diagram:



TEXT-FIG. 4. Schematic diagram of the homology between (a) Gastropod spermatocyte, (b) Gastropod nerve-cell, (c) Mammalian sympathetic nerve-cell (according to Gatenby and others).

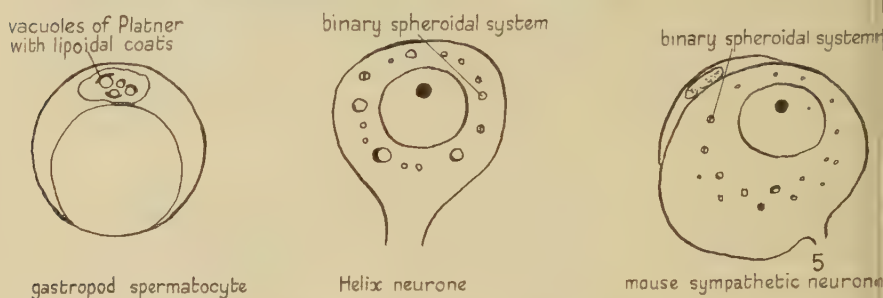
On the other hand, Parat in 1928 demonstrated that the vacuoles present in the Nebenkern and first seen by Platner (1885) in the living cell could be stained vitally with neutral red. Parat then launched his vacuome theory, which suggests that the vacuoles are, in fact, the true Golgi complement of the spermatocyte. The somewhat enigmatic lepidosomes he interprets as modified mitochondria (the 'chondriome actif'). Baker (1944) has recently re-investigated this problem and has shown that the vacuome of Parat has an external skin or coat consisting of lipines, and he identifies the binary system so formed as the Golgi material.

The author (Thomas, 1947) has previously made a detailed study of the invertebrate (molluscan) neurone with the phase-contrast microscope, controlling the observations with the newer techniques of Baker (sudan black) and Worley (methylene blue), and has put forward facts which strongly suggest that the earlier views identifying the dictyosome as the Golgi material are mistaken. Instead the cell has been shown to possess a well-marked system of spheroids possessing lipoidal capsules and stainable vitally with neutral red and methylene blue. These systems are unmistakably homologous to the spheroid bodies of the vertebrate sympathetic cell described in this paper.

Although it seems that further work is required on the problem of the

Nebenkern of gastropod spermatocytes, it is now reasonably certain that a strictly homologous series of binary spheroidal bodies exists in invertebrate and vertebrate neurones and that these bodies are, in fact, the true Golgi material of the neurone. Further, these Golgi systems would appear homologous to the vacuoles of Platner of the invertebrate spermatocyte together with the lipoidal coat to the vacuole.

The following diagram expresses this new interpretation of the homology



TEXT-FIG. 5. Schematic diagram of the author's conception of the homology between (a) *Gastropod spermatocyte*, (b) *Gastropod nerve-cell*, (c) *Mammalian sympathetic nerve-cell*.

It must be stressed that these views preclude any recognition whatsoever of a classical Golgi network as a performed living structure within the neurone. Instead this network is claimed as an artificial distortion of two separate categories of real structures—the mitochondria and the spheroid bodies.

The spheroid bodies which can be clearly followed through from the living cell to the fixed and impregnated preparation are the sole contributors to the Golgi complement of both the vertebrate sympathetic neurone and the invertebrate cerebral ganglion cells.

It would appear better to abandon the word 'Golgi apparatus' or 'Golgi material'. If a dispersed system of binary spheroids can be shown to be of general occurrence in cells, as may well be the case, a new cell nomenclature is clearly required. However, for the time being we must use for convenience such expressions as 'true Golgi complement', while bearing in mind that the object named by Golgi in nerve-cells consisted not of the binary spheroids only, but of the mitochondria and of irregular depositions of silver as well.

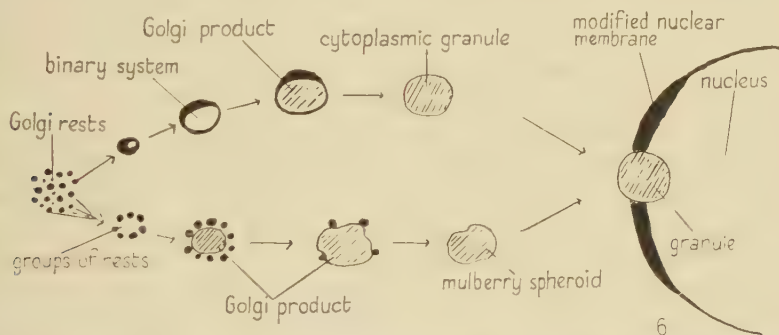
Intraneuronal Granules and the Problem of Neurosecretion

Within recent years a number of workers (Scharrer, 1940; Palay, 1943; *et al.*) have put forward the thesis that nerve-cells in certain situations can in addition to their purely conductive capacity elaborate and discharge 'neurohormones' into the blood-stream. Recently Lennette and Scharrer (1946), studying the sympathetic nerve-cells of the monkey, have demonstrated the presence of granules, remaining in the cytoplasm after celloidin embedding and hence thought to be protein in nature, which may be interpreted as a secretion antecedent. As they failed to produce evidence of a secretion cycle

they deemed that their material was not favourable for a cytological study of the problem.

In my paraffin-embedded material granules with precisely the same morphogenic and tinctorial properties occur widely throughout the tissue. Further, it has been shown that these granules are intimately associated with the Golgi spheroids. The smallest granules appear to be formed within the lipoidal pellicle of a single Golgi system for it can be shown that they are at first completely covered with a sudanophil skin. This finding is in exact accord with the observations of Hirsch and others who have shown that in exocrine gland cells, e.g. the pancreas, the secretion antecedents always make their first appearance in this situation and later emerge from the pellicle as free and independent zymogen granules.

The large irregular 'mulberry' granules appear to be formed by the concerted efforts of a group of Golgi Rests. In this case the individual Rests remain as independent small sudanophil bodies and it is possible that each contributes its quota of Golgi product to form collectively the 'mulberry' granule. Groups of small Rests are frequently found in the Helly-fixed material either without or associated with granules of varying size. Text-fig. 6 illustrates in a schematic way these two possible mechanisms for the formation of Golgi product in the neurone:



TEXT-FIG. 6. Schematic diagram to show the two possible mechanisms for the production of Golgi product within sympathetic neurones. The product is transported to the nucleus.

When the Golgi material is in contact with the nucleus the preparations resemble some of the stages of nucleolar emission reported in ova during yolk formation in *Saccocirrus* (Gatenby, 1922) and *Diemyctylus* (Saguchi, 1932). I wish to thank Professor J. Brönte Gatenby for allowing me to examine some of his preparations of *Saccocirrus* and to compare them with my own material. Nucleolar emission has also been described by Carlier (1902) in liver, gastric epithelium, and salivary gland cells of rat, cat, and newt, and by Page May and Walker (1908) in nerve-cells of mammals. After a careful study of the latter paper I have concluded that the phenomena met with in my sections are altogether different. Nuclear protrusions as shown in Page May and Walker's figs. 11 and 12 do not occur in my material;

instead the granules of Golgi material appear to apply themselves to the undeformed nuclear membrane and to spread out in a fan-shaped manner over its surface, contributing to the characteristic thickened area of nuclear membrane in the zone of contact (Plate II, figs. 4, 5, 6).

As the granules survive treatment with the alcohols and xylene during the embedding processes and further do not themselves colour with sudan black it is likely that they are of a protein nature. Further, they give a weak xanthoproteic reaction.

Their reaction with the nucleus is a very curious one. The whole appearance suggests, although it cannot be proved, that this material originally formed within the Golgi systems migrates to the nucleus and is absorbed by it.

It might be argued that the converse may well be the case and that the material has a nuclear origin and is extruded from the nucleus into the cytoplasm, as Caspersson (1939) and others have shown is the case for particles of ribose nucleic acid.

The following points can be made in favour of the former views:

1. The material, whatever its exact chemical nature may be, gives a negative Feulgen reaction and is not stained by pyronin, so it seems certain that we are not dealing with either desoxy-ribose or ribose nucleic acid.

2. The nerve-cell has been shown to contain a dispersed system of Golgi spheroids exactly comparable to the systems that Hirsch and others have shown to exist in normal glandular cells, wherein all secretion antecedents originate within the chromophobe portion of the Golgi system and are finally set free within the cytoplasm. In the vertebrate sympathetic cell, as is the case with the molluscan neurone, we can demonstrate all the stages typical of a secretory cycle of Golgi origin. In *Helix* neurones the Golgi product tends to be fatty (the granules of Legendre) whereas in the sympathetic neurone of mammals protein products predominate.

In brief, a secretion cycle can be demonstrated for the cytoplasmic origin of these granules quite apart from any suggestion of their being of nuclear origin.

The Golgi products described in this study are clearly identical with the 'secretion antecedents' claimed by Scharer and others who subscribe to the neurosecretion hypothesis. The observations concerning the nuclear absorption of these granules may well cast some light on the problem of neurosecretion in general.

It must be admitted that, however interesting these observations and conjectures may be, the central problem of the functional significance of the material of the neurone must remain unsolved until direct experimental evidence can be brought to bear on the problem.

It is the author's hope that these newer concepts of the cytology of the neurone originally proposed by Baker and expanded in this study will bear fruit in the hands of workers generally concerned with problems of nervous physiology and pathology.

In conclusion I wish to thank Dr. J. R. Baker for his ever-ready help and encouragement throughout this study and Prof. A. C. Hardy, F.R.S., for his help in preparing the text. Also to Mr. Humphrey Leach of the Physiology Dept., Oxford, and Prof. J. Brönte Gatenby, Dublin, for helpful criticism and advice.

SUMMARY

1. Evidence is put forward in support of the view that the Golgi complement of the vertebrate sympathetic neurone consists of a dispersed system of spherical bodies. These bodies can be studied in living cells and with vital dyes and each consists of a neutral red staining core (the vacuome of Parat) enveloped in a lipoidal sheath.

2. The classical Golgi body is shown to be an artifact produced within the cell. With the osmium techniques the spheroid bodies together with the mitochondria form a framework which serves as a centre for a non-specific deposition of metallic particles.

3. The Golgi spheroids exhibit a secretion cycle with the formation of a granular product. The granules are identified with the 'neurosecretion' granules of Scharrer.

4. These granules appear to be transported to the nucleus of the cell and there to be absorbed. This observed interrelationship between nucleus and Golgi product is discussed.

DESCRIPTION OF PLATES

PLATE I

FIG. 1. A photomicrograph of a sympathetic neurone fixed in Helly's fluid, post-chromed and coloured with sudan black. Two large spheroids with lipoidal pellicles contain mulberry granules (unstained). Some smaller systems are also shown.

FIG. 2. Camera-lucida drawing of the same cell.

FIG. 3. Camera-lucida drawing of a sympathetic neurone 12 hours after the commencement of the Mann-Kopsch technique. Only the pellicles of the spheroid systems are blackened.

FIG. 4. Camera-lucida drawing of a sympathetic neurone 36 hours after the commencement of the Mann-Kopsch technique. The spheroids are mostly blackened throughout; in addition numerous filamentous mitochondria have become blackened by the osmium.

FIG. 5. Camera-lucida drawing of a sympathetic neurone 3 days after the commencement of the Mann-Kopsch technique. The mitochondria are thickened and anastomose. Some of the spheroids have become incorporated on the strands so formed. The cell cytoplasm shows some general blackening.

FIG. 6. Camera-lucida drawing of a sympathetic neurone 6 days after the commencement of the Mann-Kopsch technique. The classical Golgi network is now formed. Individual spheroids are still recognizable. The cell cytoplasm shows a general non-specific deposit of osmium particles more especially around the network.

PLATE II

All photomicrographs were taken with the technique of tube length extension described by Dr. J. R. Baker in a special article on photomicrography in the *Journal of the Royal Microscopical Society*, vol. 62, p. 112. A Reichert 2-mm. fluorite immersion lens and Watson Holos eyepiece 14× were employed for Figs. 1, 2, 3, 4, and 5; for Fig. 6 a Zeiss 2-mm. apochromat with 10× compensating ocular, and for Fig. 7 a Zeiss 1.5-mm. apochromat with 10× compensating ocular.

FIG. 1. Two sympathetic neurones coloured with sudan black following Helly's fixation with post-chroming. Each cell contains one large spheroid system with sudanophil pellicle A and B.

FIG. 2. The same cells after removal of the sudan and restaining with the Azan stain. The cores of the two spheroids A and B contain azocarmine-staining granules.

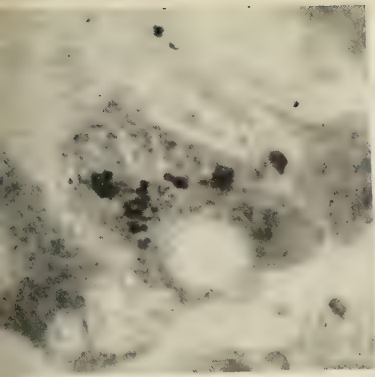
FIG. 3. Large 'mulberry' granules in a sympathetic neurone of rabbit. Helly fixation post-chromed and stained with the Azan stain.

FIGS. 4, 5, 6. Sympathetic neurone of rabbit with azocarmine-staining granule applied to the nucleus. Helly fixation post-chromed. Azan stain.

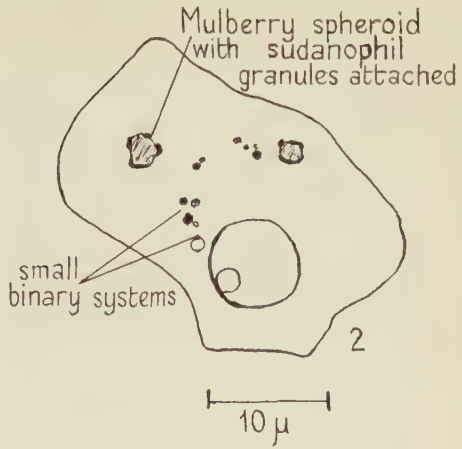
FIG. 7. A sympathetic neurone of rabbit with two nuclei. One azocarmine-staining granule in the cytoplasm. The nucleolus of the right-hand nucleus appears applied to the nuclear membrane from within. (A similar appearance to the 'Anlagerung' of Bargmann, 1948.) Helly fixation, post-chromed. Azan stain.

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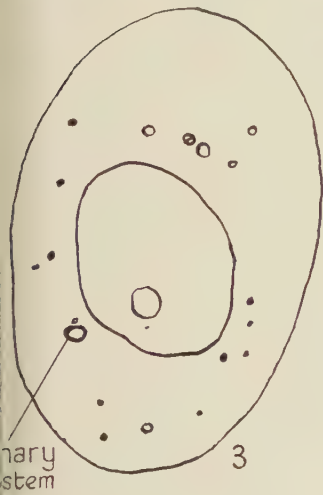
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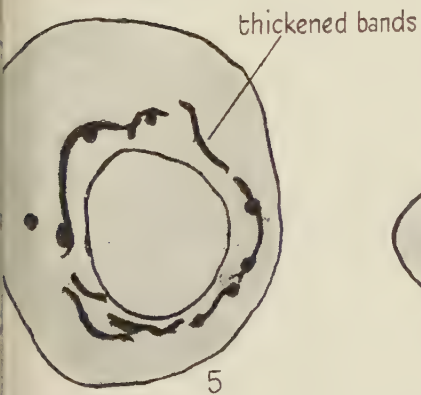
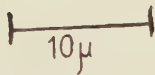
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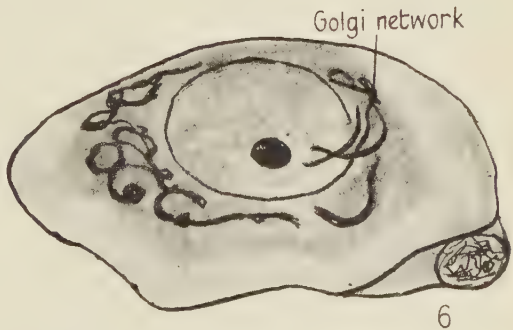
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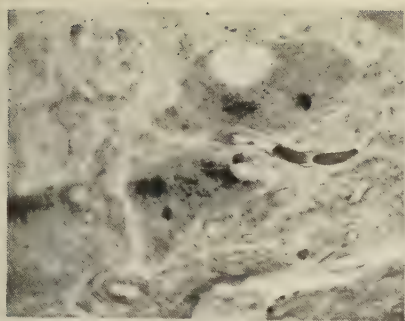
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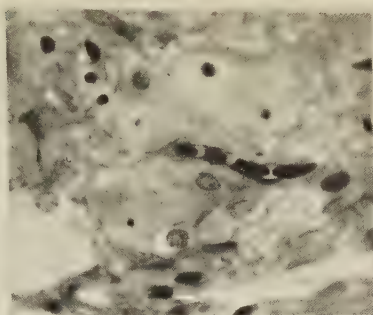
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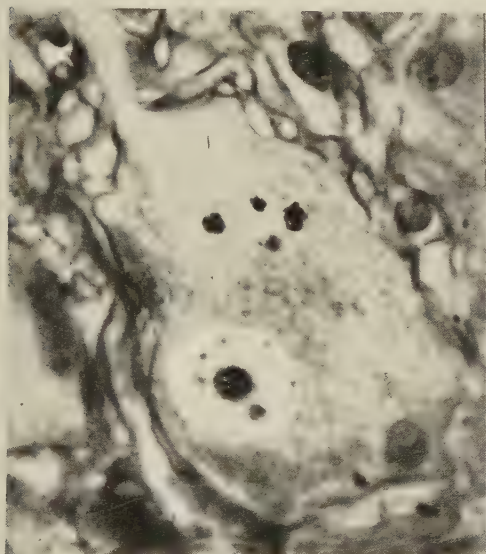
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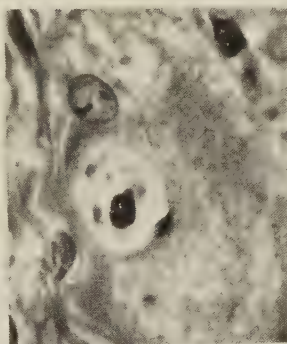
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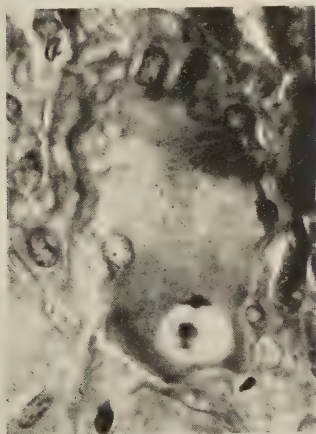
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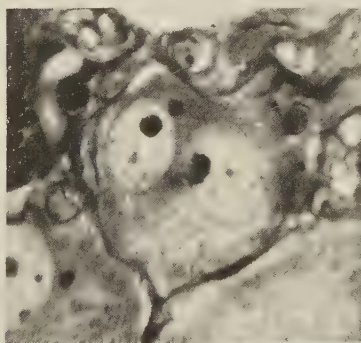
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A Simple Method for Phase-Contrast Microscopy

BY

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THE method described in this paper enables anyone to use phase-contrast microscopy without waiting to obtain special objectives or condensers.

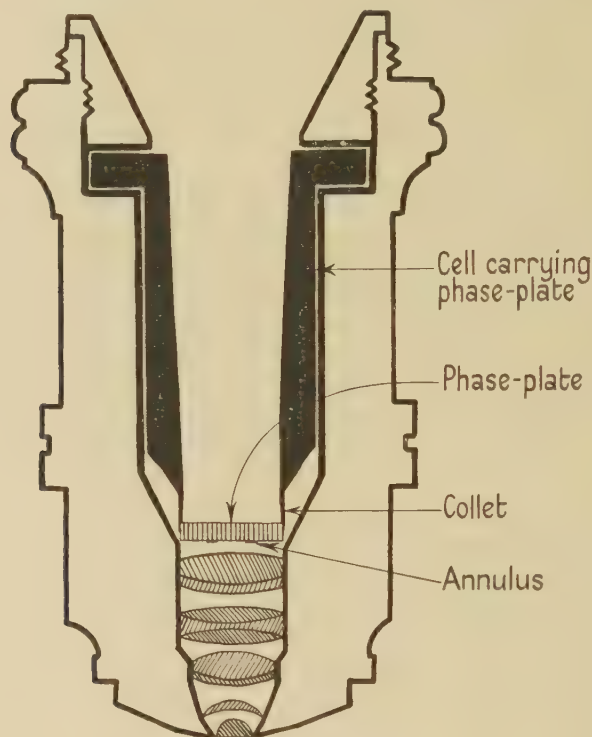
The phase-contrast method is particularly valuable in the examination of living cells with high powers. The most perfect lenses for studying living cells in body-fluids or saline solutions are water-immersion lenses, since these are specially corrected for the use of aqueous media below the coverslip. For this reason we shall describe the adjustment of a microscope to give the phase-contrast effect when a Zeiss 2.5 mm. apochromatic water-immersion lens of N.A. 1.25 is used. Although others will not use precisely this lens (and will in many cases probably use oil-immersion lenses instead), we think it useful to give actual measurements of the apparatus we describe, to serve as a guide.

The first thing to do is to prepare a phase-plate, which, in our method, will lie close *behind* (*above*) the back focal plane of the objective. Our method is not applicable to objectives carrying a phase-plate *in* the back focal plane. Obtain from a firm of lens-manufacturers a circle of glass, 1 mm. thick, of the same diameter as the back lens of the objective, and with the two sides optically plane and exactly parallel with one another. Our circle is 5.5 mm. in diameter. This plate will be made into a phase-plate, but it is necessary first to construct a special cell to hold it in position, close behind the back lens of the objective. In our apparatus the distance between the back lens and the phase-plate is 0.77 mm. The cell that maintains the plate in this position in the objective serves also to hold it while the annulus is being fashioned on its surface, as will be described below.

The cell in our apparatus (see Text-fig. 1) has the general appearance of a funnel-stop used in dark-ground illumination, but is carefully designed in such a way that it does not reduce the N.A. of the objective. It is essentially a hollow cylinder, holding the phase-plate at its lower end and secured to the objective at its upper end. The manner in which the back lens of the objective is mounted may necessitate making the walls of the cell at the phase-plate end very thin; but this is a desirable feature, for these walls by being slit can function as a spring chuck or collet, thus permitting the changing of plates.

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Most objectives have a screw-in stop in the form of a ring at the back end of the mount, and if the cell is made so as to occupy the whole of the space inside, with proper clearance for the phase-plate, the ring provides a convenient method for holding it in. Slots must be cut at several places at the mouth on the phase-plate end of the cell, thus forming spring jaws. A seating must be turned out inside the mouth, at a depth of approximately half the thickness of the plate. This shouldered seating ensures that the plate is held square to the



TEXT-FIG. 1. Longitudinal section of an immersion objective carrying a phase-plate. (The thickness of the annulus on the phase-plate has been greatly exaggerated, because it would otherwise be invisible in side-view.)

optical axis. As it is desirable to be able to change phase-plates conveniently and without causing damage, the jaws should be made very slightly bell mouthed so as to avoid chipping the edges of the glass, which is very prone to fracture if pressure is applied at one point.

The use of another simpler cell will make the insertion of the plate into the holding cell much easier and will avoid the risk of chipped edges caused by handling with forceps. This merely consists of a small block of metal with a hole turned out and shouldered, in which the plate can rest freely to a depth of less than half its thickness. Place the plate into this recess with the

bloomed surface downwards and load into the proper cell by inverting the latter over it and gently pressing until seated squarely in position.

To make the annulus, a uniform layer of 'bloom' must first be deposited on the whole of one side of the plate. Send the glass plate to a firm that provides a 'blooming' service for photographic lenses (e.g. Messrs. Pullin Optical Co., Ltd., Phoenix Works, Great West Road, Brentford, Middlesex). Since the thickness of bloom is not always exactly the same, it is a good plan to have several plates bloomed, and to find which one works best in practice with particular objects.

Our phase-plates are of the kind called 'A—' by Bennett (1946): that is to say, the annulus is raised above the surface of the glass, and darkened by a slight deposit of opaque material above the bloom. To make this type of phase-plate, the bloom must be completely removed everywhere except in the annulus itself. Remount it in the cell. A very simple form of lathe can be adapted from the ordinary turn-table used in mounting microscopical slides. There must be no free play in the bearing of the revolving disc, which should be well lubricated. In the centre of the disc, fix a simple chuck by means of sealing-wax or even plasticine. This chuck is easily made from $\frac{1}{8}$ -in. walled brass tubing, $\frac{5}{8}$ in. long and $1\frac{3}{8}$ in. in diameter, with three 4-B.A. screws tapped through to a common centre, equally spaced near one end of the tube. Mount this on the disc with the centring screws uppermost. Mark one of the screws and also the cell, so that the latter may be taken out and replaced in the chuck without much re-centring. By trial and error the phase-plate in its cell must be accurately centred in the chuck by adjustment of the screws, so that no lateral movement is perceptible as the disc is revolved. This operation and the turning off of the coating described later, require the use of a dissecting binocular, preferably of the long arm type, though it might be possible to manage with a lens fixed to a stand. Final centring requires very careful manipulation of the screws and may be helped by holding a needle (not by hand) close to the edge of the plate as it revolves. Incorrect centring causes the plate to move to and from the needle.

Originally the coating was scraped off by holding in the hand a very fine chisel-pointed needle, which was applied to the surface as it revolved. A simple device was latterly used, however, which gave precision-control of this operation and is strongly recommended. One requires a piece of wood to fit on top of the hand-rest of the slide-mounting turn-table, of such thickness that it is not higher than the phase-plate on the disc. Cut from thin sheet tin an L-piece $\frac{1}{2}$ in. wide, with arms 9 in. and $2\frac{1}{2}$ in. long. Bore a hole through the angle of the L. Pass a nail or screw through this hole and thus attach the L-piece to the wood in such a way that it swivels without play. The long arm of the L must point to the left, and the short one forwards, towards the middle of the turn-table. To make the scraping tool, grind a needle on an oil-stone to a fine chisel-point and fix it with plasticine to the end of the short arm, pointing it downwards at an angle of approximately 45° . Move and bend the short arm of the L-piece so that the scraper is about $\frac{1}{8}$ in. above the phase-

plate, thus allowing the forefinger to press it in contact with the surface of the plate; the spring tension lifts it off when pressure is released. The disc should be rotated anticlockwise and the scraping action commenced at the nine o'clock position. The left hand controls the long arm and thus slowly feeds the scraper across the surface of the phase-plate, while the forefinger of the right hand applies the pressure. The long lever effect permits precise control with comparative ease of operation.

Under the binoculars the scraped-off coating is plainly visible as a fine powder, making it quite easy to watch the process. With occasional wiping with a very soft brush, any part of the coating not properly removed can be seen at once. To scrape off the centre portion, begin at the centre of the plate, moving the scraper towards the nine o'clock position until the desired width of annulus is left.

Our annulus is 2.58 mm. in outer diameter and 1.52 mm. in inner diameter; it follows that the annulus is 0.53 mm. wide.

Needless to say, finger-marks are ruinous to results, and it is advisable to polish the back (unbloomed) surface of the plate thoroughly before it is placed in the cell.

In order to balance the direct light coming through the annulus with that of the diffracted light, carbon must be deposited on the annulus to reduce its transmission. By using a small flame, such as a cigarette lighter, with some xylene or benzene in the fuel, the plate can be smoked gradually to the desired density. Avoid overheating by occasional cooling to prevent the coating becoming temporarily soft. The density of the carbon of our best annulus has not yet been measured properly, but as a guide, it is between 1 and 1.5 photographic density, which is the equivalent of transmission of 10 to 3 per cent. The cell is taken out of the chuck, so that the rate of deposition of carbon can be watched by holding to the light. Replace in the chuck and centre accurately as before, remembering that the carbon is removed by the slightest touch. The carbon must now be removed from the clear glass, leaving it on the annulus only. Repeat the scraping technique to do this, but replace the needle with clean smooth-textured paper, cut to a fine tapering point. Examine the point carefully and remove projecting cellulose fibres. Recut the point if necessary, as the fibres are quite uncontrollable and will tend to remove carbon beyond the limit required. The removed carbon tends to build up into isolated heaps, which may be gently blown away with a pipette if care is taken that they do not touch the carbon on the annulus by being blown across its surface. The entire surface may be wiped clean from carbon with old clean linen if smoking has to be repeated.

The phase-plate, still held in its cell, is now to be placed in the objective. The side of the plate carrying the annulus will be downwards (that is, towards the back lens of the objective), as shown in Text-fig. 1. The whole optical system (except the eyepiece) is shown diagrammatically in Text-fig. 2.

A microscope-board is required to hold the lamp, illuminating-annulus and microscope in correct alinement. Obtain a suitable piece of wood about

100 cm. long and 25 cm. wide, and make sockets at one end of it into which the feet of the microscope will fit, in such a way that it will always be held in exactly the same position. Incline the microscope at a comfortable angle, and make arrangements whereby it can be inclined at exactly this angle whenever it is desired to use the phase-contrast method.

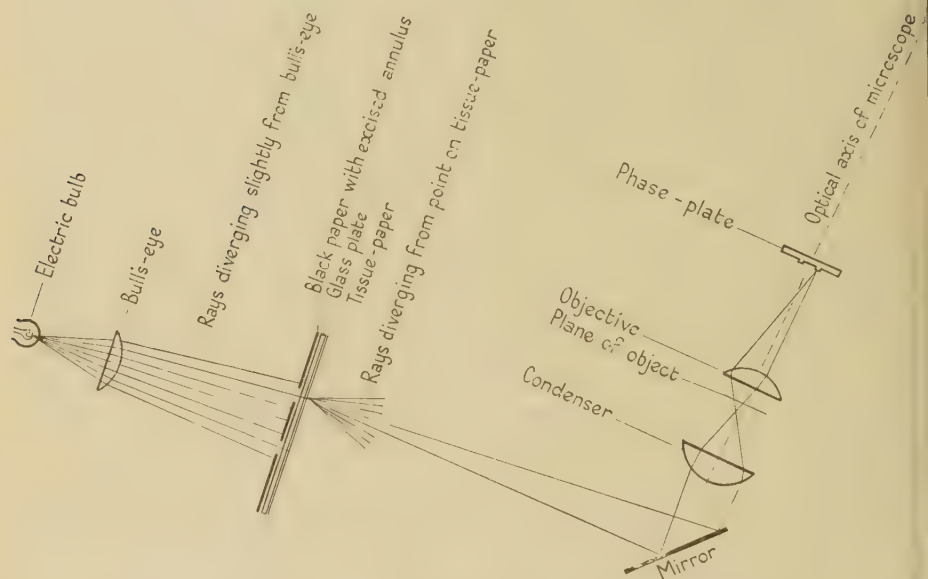
The primary source of light must be rather intense. A 6-volt filament-bulb of the motor-car headlight type is suitable. It must be contained in a suitable housing, in which a focusing bull's eye must be incorporated. Screw the lamp-stand into the far end of the board from the microscope in such a position that perpendiculars dropped from the bulb and from the centre of the bull's eye would strike the board at the same distance from its long edge as would a perpendicular dropped from any part of the optical axis of the microscope. Now incline the lamp-housing and focus the bull's eye so that the image of the filament is projected on to the centre of the mirror of the microscope. The lamp-housing must now be clamped firmly, as its position should never be changed again; but it will be necessary to change the focus of the bull's-eye, without changing the direction of its central beam.

Two modifications of the light must now be made, the one for use in ordinary microscopy, the other for phase-contrast. For the former, a sheet of finely ground glass about 8 cm. square is suitable. It must be held in a retort stand provided with a rectangular base in such a way that when this base is pushed up against the side of the microscope-board, the centre of the ground glass plate lies on the line joining the centre of the bull's eye to the centre of the mirror, and the plane of the ground glass is at right angles to this line. The ground glass should be about 20 cm. from the mirror. The microscope is now ready for ordinary use.

For phase-contrast microscopy a second modification of the light is required—a *bright annulus*. Prepare it as follows. Take a piece of black paper about 8 cm. square, and cut out a circle 39 mm. in diameter. Stick the outer piece of black paper to a square sheet of glass of the same size. Now trim down the circle of black paper to a diameter of 27.5 mm., and stick it on to the same sheet of glass in such a way as to leave an annulus approximately 5.8 mm. wide between the two. It is a good plan to put four dots of Indian ink on the glass in the annulus, N., S., E., and W., as these will help in focusing the condenser at a later stage. Now take a square of tracing-paper, of the same size as the sheet of glass, damp it all over with medicinal paraffin, and apply it to the side of the glass that does not carry the black paper, carefully avoiding the inclusion of any air-bubbles. Fix this secondary source of light in a retort stand and place it in exactly the same position as that in which the ground glass stood before. The side of the glass carrying the tracing-paper should face towards the microscope. A drop of medicinal paraffin should be applied to the tracing-paper occasionally, if there is any tendency for it to dry up. (We have found tracing-paper damped with paraffin preferable to ground glass for this particular purpose.)

Everything is now ready for use. A well-corrected condenser, of medium

power, such as the Watson 'Parachromatic' (0.29 in. focus) should be used. Place some living cells in saline on a slide, cover, and place the slide on the microscope. Using the ground glass as the secondary source of light, focus the low-power objective on the cells. Now focus the condenser of the microscope in such a way that a pencil held against the ground glass is seen in focus at the same time as the cells. Replace the low-power objective by the water-immersion carrying the phase-plate, and focus it carefully on a cell. Now move the slide slightly, till there is no cell in the field of view. Remove the draw-tube from the microscope and screw a 3-in. objective into the bottom of it.



TEXT-FIG. 2. Diagram showing the optical arrangement described in the text. Two rays are followed from a point in the illuminating annulus to a point in the annulus on the phase plate. (The eyepiece is not represented.)

Replace it in such a position that the annulus of the phase-plate is sharply focused. Be careful not to change the focus of the microscope.

Now take away the retort-stand carrying the ground glass and replace it with that carrying the annulus. Focus the bull's eye, if necessary, so that this annulus is brightly illuminated. (The bright circle cast by the lamp should be slightly larger in diameter than the annulus.) Look through the microscope and focus the condenser very slowly downwards. When a certain position has been reached, the bright annulus will be seen in focus at the same time as the phase-plate annulus. This will happen when the image of the bright annulus thrown by the condenser of the microscope lies in the plane that constitutes the focus that is conjugate to the plane of the phase-plate placed on the other side of the objective. The condenser is now in the 'low' position.

recently described by one of us ('low-condenser illumination' (Baker, 1948)).

The object is to make the two annuli coincide. This is achieved (1) by adjusting the mirror; (2) by moving the bright annulus nearer to or farther from the mirror along the line joining the mirror to the bull's eye; and (3) by making corresponding movements of the condenser so as to keep the bright annulus in focus. The position is correct when the bright annulus coincides with the phase-plate annulus but is not quite wide enough to cover the whole of it, so that the edges of the phase-plate annulus are just seen outside and inside the image of the bright annulus. It is a very great advantage to use a stainless steel mirror instead of a glass one, so as to avoid double reflexions.

It is worth mentioning that exact focusing of the image of the illuminating annulus on the phase-plate annulus appears not to be necessary. Just as good results seem to be given if it is slightly out of focus, provided that all the direct (unscattered) light passes through the phase-plate annulus.

Now remove the 3-in. objective, replace the draw-tube, and move the slide until the cells come into view. 'Negative' phase-contrast has now been achieved: the field will appear grey, and objects of high refractive index bright. Nuclei will thus appear brighter than cytoplasm. Mitochondria and any bacteria will appear particularly bright.

When a satisfactory arrangement has been achieved, make marks on the microscope-board to enable you to bring the retort-stand (and hence the bright annulus) quickly into its correct position. With the phase-plate annulus described on pp. 353-4, and with a Watson 'Parachromatic' condenser, we find it necessary to place the bright annulus 20 cm. from the mirror.

It is a convenience to have a resistance attached to the microscope board in such a position that one may alter the intensity of the light without moving the eye from the microscope.

The method of illumination adopted bears a resemblance to that used by Burch and Stock (1942), but these authors used a straight slit instead of an annulus, and the light passed directly through it instead of being rediffused as in our system.

We thank Professor A. C. Hardy, F.R.S., for encouragement and for providing all the facilities necessary for performing this work, and for valuable criticism. Dr. W. Loos kindly visited us and gave very valuable advice on the preparation of phase-plates.

SUMMARY

A method of phase-contrast microscopy is described, not involving the use of special objectives or condensers. A method for making the phase-plate carrying a raised annulus is described. A large annular source of light is

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focused by the condenser of the microscope in a plane slightly *below* the object.
The phase-plate is placed in the conjugate focus of this plane, just above the
back lens of the objective.

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